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(71) Applicant (for all designated States except US): KABI PHARMACIA AB [SE/SE]; S-751 82 Uppsala (SE).		Published <i>With international search report.</i>	
(72) Inventors; and (75) Inventors/Applicants (for US only): NYQVIST, Håkan [SE/SE]; Eklidsvägen 20, S-146 00 Tullinge (SE). EINARSSON, Monica [SE/SE]; Spillkråksvägen 4, S-756 52 Uppsala (SE). MATTSSON, Christer [SE/SE]; Rinkebyvägen 2, S-172 37 Sundbyberg (SE).			
(54) Title: A PHARMACEUTICAL COMPOSITION CONTAINING A DEFINED LIPID SYSTEM			
(57) Abstract			
<p>The present invention relates to pharmaceutical compositions containing a defined lipid system of at least two lipid components where at least one of the lipid components is amphiphatic and polar and one is nonpolar wherein the pharmaceutically active compound is a heparin or a fragment thereof. In the compositions a water containing solvent also is included in such an amount that discrete lipid particles are present, and said compositions can be adapted to various administration forms such as rectal, oral, buccal, transdermal, etc.</p>			

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A PHARMACEUTICAL COMPOSITION CONTAINING A DEFINED LIPID SYSTEM.

Field of invention

The present invention relates to pharmaceutical compositions that contain a defined lipid system of at least two lipid components where at least one of the lipid components is amphiphatic and polar and one is non polar, wherein the pharmaceutically active compound is a heparin, a fragment or derivatives thereof. The compositions further contain an aqueous solvent in an amount such that discrete lipid particles are present and said compositions can be adapted to various administration forms such as rectal, oral, buccal, sublingual, transdermal etc.

Description of the invention

This invention is based on a defined lipid system, described in the Swedish patent application SE 9003100-6, of at least two lipid components chosen from classes of different polarity, in which at least one of the compounds is amphiphatic and polar and one is non polar.

The amphiphatic and polar compound is bilayer forming and discrete lipid particles are formed spontaneously from the lipid system when it interacts with an excess amount of water or water containing solvents.

A defined lipid component is a lipid whose chemical composition is known and controlled. This will be explained more in detail below and in the Examples.

The property "bilayer forming" is a well-known physical parameter and can be established readily by suitable physicochemical methods (e.g. surface balance method). And the formed discrete lipid particles can be established by physical and/or chemical methods, such as microscopy using polarized light, image analysis or diffraction methods.

The variation in the lipid composition provides the control mechanism by means of which lipid particles are formed and thereby the rate of the lipid particle formation which will serve as a controlling factor for either immediate or sustained release of the entrapped or associated bioactive materials.

The following definitions are used:

lipids - a general term for natural or synthetic compounds consisting of acyl carriers, such as glycerol, sphingosine, cholesterol, and others or derivatives thereof, to which one or more fatty acids are or can be linked. Also similar molecules that contains a substantial hydrocarbon portion may be included.

The lipids used for the lipid particle forming systems can be grouped into different lipid classes, dependent on their polarity, namely:

non polar lipid classes - have no polar head groups or a hydrophilic part which is so small or so sterically hindered that it cannot interact with water. Examples of non polar constituents are hydrocarbons, or non-swelling amphiphiles, such as mono-, di- and triacylglycerols, cholesterol, fatty alcohols or cholesterol esters.

polar lipid classes - such as phospholipids or glycolipids, which have surface solubility due to a significant polar constituent. Depending on their specific interactions with water, they are further subdivided into the categories of swelling and soluble amphiphiles respectively.

amphiphatic or amphiphilic lipid classes - such as phospholipids and glycolipids, having surface activity,

bilayer forming lipid classes - amphiphatic lipids, such as PC (phosphatidylcholine), sphingomyelin, PI (phosphatidylinositol) or PE (phosphaditylethanolamine) with a molecular geometry that preferentially forms bilayer structures in the presence of water.

The lipids used for the carrier systems according to the present invention consist of a mixture of lipid classes characterized by their different polarities. Polar lipids, such as phospholipids or glycolipids, and non polar lipids, such as mono-, di- and triglycerides, are the main constituents in the system although sterols, such as cholesterol, fatty acids, fatty alcohols and esters thereof as well as other lipid classes may be also be used. This well defined mixture of lipids from different classes as defined above, should not be confused with commercial products such as soybean oil, maize oil or soy lecithin and egg lecithin. To obtain the well defined lipid classes the commercial oil is fractionated and then the different lipids classes are

admixed as is explained in more detail in the Examples below. Another way to obtain well defined lipid classes is to use synthetically produced lipids.

Furthermore, derivatives of lipids may also be used in combination with the above mentioned lipids. One examples of this is polyethylene glycol coupled to phosphatidylethanolamine, which has shown to prolong the circulation time of liposomes after injection in the blood stream. Another example of such a derivative is palmitoylcarnitine, which acts as an absorption enhancer for bioactive substances in the gut.

In a suitable way of initiating the formation of the lipid carrier system, the bioactive substance is admixed to a selected lipid, followed by admixing of a lipid of a different polarity. This polar/non-polar alteration may be continued for as many cycles as necessary in the specific case, involving a range of lipids with different polarities.

The preferred way of incorporation of a bioactive substance into the lipid carrier system is to admix the bioactive substance to amphiphilic lipids in order to create a homogenous formulation, where the amount of amphiphilic lipids generally are in the total range of 1-90% w/w. Such an amphiphilic lipid shall be capable of spontaneous bilayer formation. Examples thereof are amphiphilic and polar lipid classes, such as phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol or phosphatidylserine or mixtures thereof.

The design of the lipid carrier system includes not only the proper selection and/or combination of lipid classes, tailor-made for the solubilization of each bioactive substance, but also the proper selection of the distribution of fatty acids, i.e. the acyl groups attached to the lipid classes used. Variation of the acyl groups gives different physiochemical properties as will be seen in the examples below.

The rate by which lipid particles are formed from the system in a given aqueous environment can be influenced and controlled by varying the geometrical shape of the main bilayer forming lipid class, i.e. the effective head group area in relation to the steric conformation of the hydrocarbon tails.

A second way of influencing and controlling the formation of lipid particles is by varying the structure, thus the fluidity, of the hydrocarbon chains in the non polar part of the lipid system. This will influence the rate of interaction of the endogenous amphiphatic lipids and the exogenous aqueous medium.

Detailed description of the invention

According to the present invention the aforescribed defined lipid system is utilized in a pharmaceutical composition that contains a heparin, a fragment or derivatives thereof with a water containing solvent.

The expression "a heparin" denotes any fraction or class of heparin from natural, biosynthetic, synthetic or genetically engineered sources, as well as any derivative i. e. heparin esters and "fragment thereof" denotes any low molecular weight fragment of heparin or their derivatives including chemically modified synthetic heparin-like oligosaccharides.

In many non-parenteral administration forms considerable difficulties have been experienced in overcoming the poor bioabsorption of heparins.

An object of this invention is to solve this problem by providing a flexible composition based on the mentioned lipid system.

Another object of the invention is to provide a composition well-suitable for convenient forms of long-term self administration of heparins as alternatives to parenteral administration.

Such a composition can be used for the manufacture of preparations adapted for an oral, rectal, buccal, sublingual, nasal, subcutaneous or any transmembrane or transdermal administration, by adding suitable vehicles, solvents or carriers for each appropriate administration route.

The amphiphatic and polar compound is preferably phosphatidylcholine and the non polar lipid is preferably chosen from mono-, di- or triglycerides.

The amount of water in the composition should be of such significance that discrete lipid particles are formed.

This condition has to be tested for each lipid system, because the actual limit for the system to form lipid particles is dependent on the nature and the composition of the lipid components and the nature of the bioactive component to be administered by the system.

The most important considerations when choosing the components of the lipid system are the constitution of the lipids and the polarity of the bioactive drug to be incorporated in the system.

The absorption can be controlled, by varying the chain length of the glycerides between six to eighteen carbon atoms, preferably between six to twelve carbon atoms. The choice of polarity and the choice of differently charged groups of the constituents can also influence the lipid particle formation.

The lipid particles may be a mixed population containing different mono-, bi- and multilayered structures such as micelles, liposomes, and higher multilayered structures.

There are a number of methods well known in the art for controlling the population both in size range and in structure such as sonication, freeze-thawing controlled stirring and various size range influencing equipments. The need of such devices is to be considered separately for each administration form and each therapeutically active compound to be delivered with the composition.

The composition of the lipid particle population is also influenced by the choice of lipid material in terms of polarity, carbon chain length(s) and other factors as discussed above.

In a preferred composition according to the invention the compound is a heparin or a fragment thereof and the lipid system comprises a phosphatidylcholine as an amphiphilic compound and a monoglyceride as the non polar component.

The amount of amphiphilic compounds in the composition may be within the range of 1-90% (w/w) of the lipid system, preferably between 1-50% (w/w) and most preferably between 5-50%(w/w) with respect to the lipid system.

A preferred heparin fragment is Fragmin®, which is a low molecular weight heparin fragment prepared by Kabi Pharmacia from porcine heparin by a controlled nitrous acid depolymerisation process, see EP 14184.

Fragmin® may be in the form of a solution or a suspension, when added to or mixed with the other components of the composition. It may also be admixed with the essentially water free lipid matrix before the other constituents of the compositions are added.

Fragmin® is very readily soluble in water and such aqueous solutions are considered to be stable.

The properties of Fragmin® makes possible numerous preparations based upon the defined lipid system with the addition of suitable excipients.

Such preparations may be adapted for rectal use in the form of foams, clysmas, capsules and suppositories or different preparations specially manufactured for oral, buccal, sublingual, nasal, transdermal and subcutaneous administration or for administration through mucous membranes in general. The inventive composition containing Fragmin® and defined lipids is also suitable for the manufacture of a depot preparation for obtaining sustained release for e.g. subcutaneous administration or for application to a porous polymeric matrice with mucoadhesive properties for e.g. buccal administration.

The preparations are useful for treating and/or preventing a wide variety of pathological processes such as thromboembolic diseases, pre-infarctional angina, coronary heart diseases, inflammatory diseases, thrombophlebitis, autoimmune diseases, arteriosclerosis or for treatment of metastasis or angiogenesis related diseases.

Any adaptation of the compositions for the above-mentioned administration routes for any mentioned treatment will be obvious to persons skilled in this art.

The total dosage of Fragmin® in the above-mentioned administration forms can thus be very high and is limited only by the bioavailability and by that which is therapeutically or clinically appropriate to administer and that which is well tolerated.

Examples of clinical doses are 120 IU per kg body weight twice daily for deep venous thrombosis and for thrombosis prophylaxis (for low risk patients) 2500 IU per day or 5000 IU per day (for high risk patients).

For example, the relative amount of Fragmin® to lipids may be in the range of 1-200 (w/w), preferably 2-80 (w/w).

However a higher amount of both Fragmin® and lipids are conceivable within the context of the invention if it is clinically suitable.

If the amount of lipids will be low in a composition the enhanced absorption effect will be reduced, which may be compensated by an increased amount Fragmin® in order to obtain the same serum levels after administration.

This broad concentration range, which makes possible Fragmin® to lipid in a relative amount of up to 200 (w/w) indicates the flexibility of the components to be adapted to a composition for suitable dosage forms to be delivered to different types of patients.

The solubility and stability of Fragmin® facilitates the adaption of the composition to a suitable administration form and furthermore there is a strong evidence that it might have a stabilising influence on the lipid

particle population of the composition, probably due to its high negative charge density.

The following examples, which shall not be considered to limiting the scope of the invention, show Fragmin® in compositions with lipids defined in the examples in the proportions of 25% phosphatidylcholine as the amphiphilic compound and 75% monoglyceride as the non polar compound.

The compositions have been administered rectally and intraduodenally in-vivo with various amounts of additional water based solvent.

In the intraduodenally applied compositions the water content may vary from 1 to 4 by parts of weight with respect to the lipid constituents of the composition.

The relative amounts of Fragmin® to lipid tested were from 2 up to 200 (w/w).

Examples 1-5 below illustrate the variation of the lipid constituents of the lipid system, in the absence of bioactive compounds, by selection of lipids and combinations thereof without limiting the scope of protection.

Examples 6-7 show the preparation of compositions which contain the defined lipid system consisting of a phosphatidylcholine, glycerol esters and Fragmin®. These compositions does not initially comprise a water based solvent in an amount such that lipid particle formation direct will occur directly and spontaneously.

Example 8 shows the animal model used in the experiments in the following examples and the bioavailability of Fragmin® after an intraduodenal or rectal administration in the absence of the lipid system.

Example 9 shows that the intraduodenal absorption of Fragmin® is significantly increased when included in the lipid system.

Example 10 shows that intraduodenal absorption is increased when external water is added before administration.

Example 11 shows that the intraduodenal absorption is also similarly enhanced when the water in example 10 is exchanged with physiological saline in the same amount.

Example 12 shows that rectal absorption of Fragmin® is significantly higher when incorporated in the lipid system as compared with Fragmin® dissolved in physiological saline, in the absence of the lipid system.

Example 13 shows that the addition of external water or physiological saline to the lipid system has a positive effect on rectal absorption.

Examples 14 and 15 show that Fragmin® need not necessarily be included in the lipid system before dissolution with water or saline, in order to be absorbed from the duodenum or the rectum.

Example 16 illustrates that the tested commercially available crude lipids give a lower rectal absorption than the defined lipids.

Example 17 is a comparison of the absorption of different Fragmin® to lipid ratios and shows that the rectal absorption is high over a broad lipid and Fragmin® concentration range.

Example 18 is a comparison of the heparin and Fragmin® absorption in preparations which contain the lipid system.

Example 19 shows that after formation of the composition, the solution can be stored at varying time lengths before administration without a significant influence of the absorption properties.

Example 20-21 shows that the Fragmin® activity is retained and its absorption is still high after prolonged storage of the composition.

The results show that the defined lipid system enhances intraduodenal absorption and that the highest absorption of intraduodenally applied Fragmin® is achieved with the highest tested water content of the composition. The tested concentrations of the components showed favourable results. However it may be possible to optimize the conditions still further.

The reasons for the enhanced absorption may be that a higher contact surface area between the composition and the intestinal membranes is obtained, but also probably because that the lipid particle population has an advantageous structural composition at the degree of dilution concerned.

The lipid particles may also protect the drug from enzymatical degradation in the intestines, both if the lipid particles carries the drug or if they are otherwise present in the solution. A large amount of lipids present may block the Fragmin® degrading enzymes and in such a case the Fragmin® might be well protected outside the lipid particles. However, when the amount lipids are low, it may be advantageous to enclose the Fragmin® within the lipid particles.

Enzyme inhibitors are a conceivable additive to the inventive compositions.

It is also noted that absorption is high, irrespective of whether Fragmin® is initially present free in the solution or incorporated in the lipid particles. Excellent absorption results are also shown after rectal administration of Fragmin® compositions with the lipid system in a wide range of present water or saline. These results are comparable with subcutaneous injections, which is the most common administration route today.

It is also shown that the defined lipid system is advantageous compared to commercial lipids in terms of controlling and stabilising the compositions as well as reproducing the absorption.

It is also observed that the size of the particles formed can vary considerably, without influencing the favourable absorption properties of Fragmin®. The compositions are shown to be equally useful after short and long periods of time after their preparation.

The compositions according to the invention also enable the heparin absorption to be enhanced, when administered with the lipid system. The defined lipid system used in the present invention is also advantageous because its well-defined constituents enable the biological absorption and also the release and solubilisation of the drug to be controlled more accurately.

This means the choice of appropriate lipid constituents from the lipid classes will enable a better control of the absorption to be achieved.

It is also noted that high absorption can be obtained even if Fragmin® is not included in the lipid system during the manufacturing, but added as a solution to a lipid composition during shaking or sonication.

In conclusion it must be considered that compositions based on the defined lipid system and heparins or heparin fragments are surprisingly favourable in terms of enhanced absorption and provide an advantageous possibility of controlling the absorption rate.

Various modifications and equivalents will be apparent to one skilled in the art and may be used in the compounds, compositions and methods of the

present invention without departing from the spirit or scope thereof, and it is therefore to be understood that the invention is not to be limited to the specific examples and embodiments herein.

EXAMPLES

EXAMPLE 1

1.25 g phospholipids from soybean (I) were added to 1.25 g of a glyceride mixture (II) and gently stirred for 12 hours at 60 °C. 2.50 g of a triglyceride (III) were then added and the total mixture was stirred for 1 hour at 60 °C.

<u>Lipid class composition (g)</u>	I	II	III	<u>Fatty acid composition of triglyceride (wt%)</u>	
Phosphatidylcholine	0.50				
Phosphatidylethanolamine	0.40				
Phosphatidylinositol	0.23				
Non polar lipids	0.12				
Monoacylglycerol		0.63			
Diacylglycerol		0.63			
Triacylglycerol			2.50	8:0 caprylate	58.5
				10:0 caprate	40.5
				12:0 laurate	0.6
				16:0 palmitate	
				18:0 stearate	
				18:1 oleate	
				18:2 linoleate	
				18:3 linolenate	
				minors	0.4
Total	1.25	1.25	2.50	Total	100

EXAMPLE 2

1.25 g phospholipids from soybean (I) were added to 1.25 g of a glyceride mixture (II) and gently stirred for 12 hours at 60 °C. 2.50 g of a triglyceride (III) were then added and the total mixture was stirred for 1 hour at 60 °C.

<u>Lipid class composition (g)</u>	I	II	III	<i>Fatty acid composition of triglyceride (wt %)</i>	
Phosphatidylcholine	0.40				
Phosphatidylethanolamine	0.35				
Phosphatidylinositol	0.18				
Phosphatic acid	0.07				
Non polar lipids	0.25				
Monocacylglycerol		0.63			
Diacylglycerol		0.63			
Triacylglycerol			2.50	8:0 caprylate	58.5
				10:0 caprate	40.5
				12:0 laurate	0.6
				16:0 palmitate	
				18:0 stearate	
				18:1 oleate	
				18:2 linoleate	
				18:3 linolenate	
				minors	0.4
Total	1.25	1.25	2.50	Total	100

EXAMPLE 3

1.25 g phospholipids from soybean (I) were added to 1.25 g of a glyceride mixture (II) and stirred gently for 12 hours at 60 °C.

<u>Lipid class composition (g)</u>	I	II
Phosphatidylcholine	0.40	
Phosphatidylethanolamine	0.35	
Phosphatidylinositol	0.18	
Neutral lipids	0.32	
Monoacylglycerol		0.63
Diacylglycerol		0.63
Triacylglycerol		
Total	1.25	1.25

EXAMPLE 4

1.25 g phospholipids from soybean (I) were added to 1.25 g of a glyceride mixture (II) and 0.16 g ethanol. The total mixture was stirred gently for 6 hours at 60 °C. 0.16 g of a triglyceride (III) was added and the total mixture is stirred for another hour at the elevated temperature.

<u>Lipid class composition (g)</u>	I	II	III
Phosphatidylcholine	0.40		
Phosphatidylethanolamine	0.35		
Phosphatidylinositol	0.18		
Neutral lipids	0.32		
Monoacylglycerol		0.63	
Diacylglycerol		0.63	
Triacylglycerol			0.16
Total	1.25	1.25	0.16

EXAMPLE 5

2.50 g phosphatidylcholine from soybean (I) and 7.50 g of a monoglyceride (II) were stirred gently for 6 hours at 60 °C. 1.25 g water were added and the stirring continued for another hour at the elevated temperature.

<u>Lipid class composition (g)</u>	I	II
Phosphatidylcholine	2.50	
Monoacylglycerol		7.50
Total	2.50	7.50

EXAMPLE 6

2.50 g phosphatidylcholine from soybean (I) and 7.50 g of a monoglyceride (II) were stirred gently for 6 hours at 60 °C. 1.25 g Fragmin® solution (120 mg/g water) was added and the stirring continued for another hour at the elevated temperature.

<u>Lipid class composition (g)</u>	<u>I</u>	<u>II</u>
Phosphatidylcholine	2.50	
Monoacylglycerol		7.50
Total	2.50	7.50

EXAMPLE 7

2.50 g phosphatidylcholine from soybean (I) and 7.50 g of a monoglyceride (II) were stirred gently at 60 °C for 6 hours. 0.625 g Fragmin® solution (120 mg/g water) was added and the stirring continued for another hour at the elevated temperature.

<u>Lipid class composition (g)</u>	I	II	<u>Fatty acid composition of</u>	
<i>monoacylglycerol (w %)</i>				
Phosphatidylcholine	2.50			
Monoacylglycerol		7.50		
			8:0 caprylate	78.4
			10:0 caprate	21.2
			12:0 laurate	0.2
			minors	0.2
Total	2.50	7.50	Total	100

The size distribution of the lipid particles formed in water at 37 °C was determined for Example 15, using a Malvern equipment. The formulation was shaken gently in water for 17 hours and then centrifuged as to separate the lipid phase from the aqueous phase. The following result was obtained.

Size	%

<1µm	36
>1µm, <2µm	60

EXAMPLE 8

Animal model

New Zealand white rabbits of both sexes, weighing 2.5-3.5 kg were used in all experiments. After fasting for 12 hours, each animal was sedated with an intravenous injection of Hypnorm 0.1 ml/kg (Janssen Pharmaceuticals, Belgium) and a subcutaneous injection of Atropine, 0.5 mg/kg (Kabi Pharmacia, Sweden). The rabbits were anaesthetized with Mebumal, 20 mg/kg. The anaesthesia was subsequently maintained as required. The animals were shaved and a medial laparotomy performed. The test compounds were then injected directly into the duodenum, after which the peritoneal cavity was closed. Rectal administration was performed by means of a syringe coupled to a plastic tube designed for use in the rectum of rabbits. The dosage was checked by weighing of the syringe and connecting tubings before and after administering the test compounds.

Blood samples (1 ml) were collected at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9, and 10 hours with the aid of catheter inserted in the ear artery and mixed with sodium citrate to a final concentration of 0.04 M. The tubes were kept on ice until centrifuged at 800 g for 10 minutes. The anti-FXa activity in plasma was determined with an initial rate assay using bovine FXa (Kabi Pharmacia) and the chromogenic substrate S-2222.

The apparent bioavailability of Fragmin® after an intraduodenal or a rectal administration is expressed as a fraction of the area under the anti-FXa activity curve i.e. (AUC intraduodenal/rectal) and (AUC_{s.c.}) the latter determined to be 5.14 ± 0.34 arbitrary units in 6 rabbits receiving a subcutaneous injection of Fragmin® at a dosage of 2 mg/kg.

Bioavailability of Fragmin® in the absence of lipids

The bioavailability of Fragmin® after an intraduodenal administration in the absence of the lipid system was tested in 4 rabbits. Two of the rabbits received Fragmin®, 25 mg/kg, as a powder included in a gelatine capsule which was placed in the duodenum at the pylorus level. The remaining two rabbits received Fragmin® dissolved in physiological saline, which was injected directly into the duodenum (total dosage 25 mg/kg). No anti-FXa activity could be detected in any of these animals over an observation period of 8-10 hours.

The bioavailability of Fragmin® after a rectal administration was tested in 3 rabbits. Fragmin® was dissolved in physiological saline and injected directly into the rectum at a dosage of 10 mg/kg. Two of these rabbits had no detectable anti-FXa activity in plasma but one showed an activity of 0.2 IU/ml for 45 minutes which corresponds to a bioavailability of less than 1%.

Conclusion. The bioavailability of Fragmin® in the absence of enhancing additives is less than 1% after an intraduodenal or a rectal administration.

EXAMPLE 9

The intraduodenal absorption of Fragmin® included in the lipid system according to Examples 5-7 was tested in 8 rabbits. The Fragmin®/lipid system was mixed with water in such an amount that discrete, individual lipid particles are formed, ten minutes before being applied into the duodenum. The composition of the mixture was as follows: 1 part (weight) Fragmin®/lipid + 1 part (weight) distilled water. The water/lipid system was shaken vigorously for 5-10 minutes and then left to stand at room temperature for another 10 minutes before being

injected into the duodenum. The concentration of Fragmin® was 2% of the lipid weight. All the rabbits received the following doses: Fragmin® 25 mg/kg, Lipids 1250 mg/kg and distilled water 1250 mg/kg. All rabbits in this experiment had detectable levels of anti-FXa activity in plasma. The maximal plasma concentration (C_{\max}) was reached within 0.5-1.5 hours and ranged between 0.37 IU/ml to 1.76 IU/ml. The average absorption in this experiment was 4.7 ± 3.0 %

Conclusion. The intraduodenal absorption of Fragmin® is significantly increased when included in the lipid system, as compared to the absence of lipids in the system.

EXAMPLE 10

This experiment was identical to the study described in Example 9 with the exception that the Fragmin®/lipid system was mixed with a greater amount of water. The composition of the mixture was as follows: 1 part (weight) Fragmin®/lipid + 4 parts (weight) distilled water. The concentration of Fragmin® was 2% of the lipid weight. All the rabbits (n=9) received the following doses: Fragmin® 25 mg/kg, Lipids 1250 mg/kg and distilled water 5000 mg/kg (= 5 ml). All rabbits in this experiment had detectable levels of anti-FXa activity in plasma. C_{\max} was reached within 0.75-1.5 hours and ranged between 0.73 IU/ml to 3.79 IU/ml. The average absorption in this experiment was 14.7 ± 5.6 %

Conclusion: The intraduodenal absorption of Fragmin® is significantly increased when the lipid system (containing Fragmin®) is mixed with water before being injected into the duodenum.

EXAMPLE 11

This experiment was identical to the study described in Example 10 with the exception that the Fragmin®/lipid system was mixed with 4 parts of physiological saline instead of the same amount of distilled water. This was done in order to study if the ionic strength of the Fragmin®/lipid/water system had any effect on the intraduodenal absorption of Fragmin®. The mixture had the following composition: 1 part (weight) Fragmin®/lipid + 4 parts (weight) physiological saline. The concentration of Fragmin® was 2% of the lipid weight. All the rabbits (n=10) received the following dosage: Fragmin® 25 mg/kg, Lipids 1250 mg/kg and physiological saline 5000 mg/kg. All rabbits in this experiment had detectable levels of anti-FXa activity in plasma. C_{\max} was reached within 0.75-1.5 hours and

ranged between 0.62 IU/ml to 2.60 IU/ml. The average absorption in this experiment was 13.4 ± 7.3 %

Conclusion: The intraduodenal absorption of Fragmin® is significantly enhanced when the Fragmin®/lipid system is mixed with physiological saline.

EXAMPLE 12

The rectal absorption of Fragmin® included in the lipid system was tested on 5 rabbits. The lipid system, without water, was injected directly into the rectum at a dose of 100 mg/kg corresponding to a Fragmin® dosage of 2 mg/kg. All rabbits in this experiment had detectable levels of anti-FXa activity in plasma. C_{max} was reached within 1.5-2.5 hours and ranged between 0.53 IU/ml to 1.98 IU/ml. The average absorption in this experiment was 67.0 ± 18.2 %

Conclusions. The rectal absorption of Fragmin® included in the lipid system without addition of an aqueous solution is dramatically higher than the rectal absorption of Fragmin® alone dissolved in physiological saline.

EXAMPLE 13

Addition of external water or physiological saline to the lipid system had a positive effect on the intraduodenal absorption (See Examples 9, 10 and 11) This experiment was carried out in order to test if a similar effect could be seen after a rectal administration. Fragmin®/lipid system was mixed with 4 parts of physiological saline as described in Example 11 ten minutes before being administered to the rectum. The composition of the mixture was as follows: 1 part (weight) Fragmin®/lipid system + 4 parts (weight) of physiological saline. The Fragmin® concentration was 2% of the lipid weight. This composition was tested in 7+7 rabbits at a Fragmin® dosage of 2 and 5 mg/kg, respectively. The lipid dosage was 100 and 250 mg/kg and the amount of physiological saline was 400 and 1000 mg/kg. The results are summarized in the table below.

DOSE (mg/kg)	AUC(IU/mlxh)	C_{max} (IU/ml)	ABSORPTION (%)
2	4.4	1.43 ± 0.51	85.6 ± 29.7
5	11.8	2.89 ± 0.78	91.6 ± 30.0

Conclusion. The rectal absorption of Fragmin® included in the lipid system and mixed with external saline was almost as high as that observed after a subcutaneous injection, which today is the most commonly used route of

administering Fragmin®. The rectal absorption of Fragmin® shows a good dose-proportionality as a 2.5-fold increase in the dosage results in a similar increase in AUC and C_{max} while the absorption rate is constant.

EXAMPLE 14

In all the experiments described above Fragmin® was included in the lipid system prior to the adding of water or physiological saline to the system. In the following experiments, exactly the same lipid system was used, but without included Fragmin®. This lipid composition, without Fragmin®, will in the following discussion be referred to as "placebo lipids"

The intraduodenal absorption of Fragmin® mixed with placebo lipids was tested on 7 rabbits. Fragmin® powder was dissolved in physiological saline to a final concentration of 5 mg/ml (0.5%). Four parts (weight) of this Fragmin® solution was mixed with 1 part (weight) of the placebo lipid. The concentration of Fragmin® in this mixture was 2% of the lipid weight, which is the same Fragmin®/lipid ratio as that in examples 11-15. The mixture was shaken vigorously for 5-10 minutes or alternatively sonicated for an appropriate length of time before being injected intraduodenally. All the rabbits received the following dosage: Fragmin® 25 mg/kg, Lipids 1250 mg/kg and distilled water 5000 mg/kg. All rabbits in this experiment had detectable levels of anti-FXa activity in plasma. The maximal plasma concentration (C_{max}) was reached within 0.5-2.0 hours and ranged between 0.53 IU/ml to 3.55 IU/ml. The average absorption in this experiment was 13 ± 8 %.

Conclusion: This experiment shows that Fragmin® need not necessarily be included in the lipid system prior to adding water, in order to be absorbed from the duodenum. A mixture of placebo lipids and water or saline, containing dissolved Fragmin®, is absorbed to a similar degree. Favourable results are obtained both by shaking and sonicating the composition.

EXAMPLE 15

The rectal absorption of Fragmin® mixed with placebo lipids was tested in 6 rabbits. The mixture was prepared as described in Example 14 and injected rectally at the following dosage: Fragmin® 2 mg/kg, Lipids 100 mg/kg and physiological saline 400 mg/ml. All rabbits in this experiment had detectable levels of anti-FXa activity in plasma. C_{max} was reached within 0.5-2.0 hours and ranged between 0.59 IU/ml to 1.95 IU/ml. The average absorption in this experiment was 64.2 ± 31 %

Conclusion: This experiment shows in accordance with Example 14 that Fragmin® need not necessarily be included initially in the lipid system in order to be absorbed from rectum. A mixture of placebo lipids and saline, containing dissolved Fragmin®, is also absorbed to a higher degree than Fragmin® without lipids. (compare with Example 13).

EXAMPLE 16

The rectal absorption of Fragmin® mixed with commercially available lipids was tested on 4 rabbits. The following commercial lipids were used: IMWITOR MG 742 (from MG Hüls AG, containing about 50% monoglycerides and 50% di- and triglycerides with acyl radicals principally derived from caprylic acid and capric acid) and the phosphatidylcholine Zigma PC (P-5394 from Sigma, containing 84% PC, 13% PE and 3% other constituents). The commercial lipids were mixed with physiological saline as described in Examples 14 and 15 and injected rectally at the following dosage: Fragmin® 2 mg/kg, Lipids 100 mg/kg and physiological saline 400 mg/ml. Two out of 4 rabbits in this experiment had no detectable levels of anti-FXa activity in plasma and two had low levels in the range of 0.13 IU/ml to 0.25 IU/ml. The average absorption in this experiment was $0.7 \pm 1.5\%$ (including those with no detectable absorption).

Conclusion: When the highly defined lipid system was exchanged for the cruder lipids from which they have been purified, a dramatic decrease in rectal absorption also was observed which demonstrates that the enhanced absorption described in a number of the Examples above is not an effect of lipids in general but can be ascribed to the effect of the defined lipid system.

EXAMPLE 17

The amount of Fragmin® in relation to the amount of lipids has in the examples described above always been 2 mg/kg body weight per 100 mg/kg body weight. The following experiment was designed in order to study rectal absorption as a function of the Fragmin®/lipid ratio. One part of the placebo lipid system was mixed with 4 parts of physiological saline containing dissolved Fragmin® as described in Example 16, but with a 16 hour storage period. A higher Fragmin®/lipid ratio was obtained by increasing the amount of Fragmin® dissolved in saline. The following compositions were tested with respect to rectal absorption:

group no	Fragmin®/lipid	mg Fragmin®/100 mg lipid	mg saline/kg
	mg/kg	ratio	

1 (n=8)	2:100	2	400
2 (n=9)	2:50	4	200
3 (n=8)	2:25	8	100
4 (n=7)	2:10	20	40
5 (n=8)	2:5	40	40*
6 (n=8)	2:2.5	80	40*
7 (n=4)	2:1	200	15*

*due to practical reasons (too small volumes) the lipid concentrations could not be kept constant.
n is the number of rabbits tested.

The following results were obtained:

Group no	Fragmin/lipid ratio	AUC	C _{max} IU/ml	Absorption %
1	2	3.0±1.3	1.1±0.4	53.2±9.4
2	4	3.6±1.1	1.1±0.4	74.3±17.3
3	8	4.6±1.5	1.6±0.5	86.0±27.2
4	20	3.2±1.4	1.3±0.4	73.5±18.1
5	40	3.7±1.2	1.3±0.6	80.1±12.9
6	80	2.0±1.1	0.6±0.3	48.5±20.9
7	200	1.0±0.6	0.3±0.2	20.7±11.4

Conclusions: All the Fragmin®/lipid compositions tested gave a high rectal absorption which indicates that the concentration of Fragmin® and lipids in the described system give an increased absorption when compared to Fragmin® without lipids. The lipid composition has an enhancing effect on the rectal absorption of Fragmin®.

EXAMPLE 18

Fragmin® is a low molecular weight heparin fragment prepared from porcine heparin by a controlled nitrous acid depolymerisation process. Several investigators have shown that heparin is poorly absorbed (< 1%) after oral or intraduodenal absorption. The aim of the present study was to investigate if the lipid system could enhance the rectal absorption of heparin. The experimental procedure was precisely the same as that described in Example 14 with the exception that Fragmin® was exchanged for heparin. Eight rabbits received the following doses: Heparin 2 mg/kg, Lipids 100 mg/kg and distilled water 400 mg/kg. All rabbits in this experiment had detectable levels of anti-FXa activity in plasma. The maximal plasma concentration (C_{max}) was reached within 0.5-2.0

hours and ranged between 0.12 IU/ml to 0.53 IU/ml. The average absorption in this experiment was $15.7\pm 8\%$.

Conclusions: The rectal absorption of heparin is significantly increased when a solution thereof is admixed with the lipid system. The degree of absorption, however, is significantly lower than that of Fragmin®, when they are compared under identical conditions. This indicates that there is a size dependency or specific interaction in the absorption pattern of molecules of heparin origin, which give possibilities to design and control the preparations to be administered.

EXAMPLE 19

All the samples described so far were injected 10 minutes after preparing the Fragmin®/lipid/water composition.

3 rabbits which received lipid/saline composition containing 4% Fragmin® were checked in order to ascertain whether or not the time lapse between preparing the composition and its administration had any influence on absorption.

The mixture was prepared as in Example 14, but in addition it was allowed to stand for 16 hours before being injected rectally in a dose of Fragmin® 2 mg/kg, lipids 50 mg/kg and physiological saline 200 mg/kg body weight. All rabbits in this experiment had detectable levels of anti-FXa activity in plasma. C_{max} was reached within 0.5-2.0 hours and ranged in between 0.72 IU/ml to 1.90 IU/ml with an average of 1.12 IU/ml. The average absorption in this experiment was about 65%.

Conclusion: The data demonstrates that after being formed, the composition, can be administered after standing for different lengths of time without significant influence on the absorption properties.

EXAMPLE 20

In vitro experiments were carried out in order to study the influence of the lipid composition on the Fragmin® anti-FXa activity in terms of time of storage. Fragmin® was incorporated into the lipid system as defined in the examples above. The concentration of Fragmin® was 2% by weight of the lipids. One part (weight) of the Fragmin®/lipid and 4 parts(weight) of distilled water were mixed by shaking and then allowed to stand for 6 weeks. The Fragmin® activity was tested regularly over this period. The initial activity 85 IU/mg (series 1) and 78 IU/mg(series 2), respectively, and the activity after 6 weeks was 96 IU/mg (series 1) and 85 IU/mg (series 2).

Conclusion: The results shows that the Fragmin® activity is retained during prolonged storage of the composition.

EXAMPLE 21

The same procedure as in Example 19 was performed on 4 rabbits with a lipid/saline composition of 2% Fragmin®. The administered dose of Fragmin® was 2 mg/kg with 100 mg/kg lipids and 200 mg/kg of saline. All animals had detectable levels of anti-Fx activity in plasma. C_{max} was reached within 0.75-1,5 h and ranged between 0.82 IU/ml and 0.86 IU/ml, with a mean value of 0.86 IU/ml. The average absorption in this experiment was about 48%.

Conclusion: The dispersion was easy to administrate, and to adjust for the calculated volumes and showed enhanced absorption of Fragmin®.

Claims

1. A pharmaceutical composition based on a defined lipid system having at least two lipid components and at least one bioactive compound characterized in that at least one of the lipid components is amphiphatic and polar and one is non polar, the bioactive compound is a heparin or a fragment or a derivative thereof; and that the composition includes a water containing solvent.
2. A composition according to claim 1 characterized in that the water containing solvent is present in amount such that individual and discrete lipid particles are formed.
3. A composition according to claims 1-2 characterized in that it is adapted for oral, rectal, buccal, sublingual, nasal, subcutaneous or transdermal use or for administration to mucous membranes and therewith contains suitable excipients for each appropriate administration route.
4. A composition according to claims 1-3 characterized in that the amphiphatic and polar lipid components are bilayer forming and chosen from phosphaditylcholine and other phospholipids and are present in an amount of 1 to 90% w/w of the lipid system and preferably in the range of 1 to 50% w/w.
5. A composition according to claims 1-4 characterized in that the non-polar lipid component is chosen from the classes of mono-, di- or triglycerides.
6. A composition according to claim 5 characterized in that the non-polar component consists either of :
 - a) monoglycerides having defined chain lengths within the range of 6 to 18 carbon atoms and/or
 - b) diglycerides having defined chain lengths within the range of 6 to 18 carbon atoms and/or
 - c) triglycerides having defined chain lengths within the range of 6 to 18 carbon atoms.

7. A composition according to claims 5 or 6 characterized in that the non-polar lipid component consists either of:

- a) a triglyceride with essentially a mixture of 8:0 caprylate and 10:0 caprate and/or
- b) a diglyceride with essentially a mixture of 8:0 caprylate and 10:0 caprate and/or
- c) a monoglyceride with essentially a mixture of 8:0 caprylate and 10:0 caprate.

8. A composition according to any of claims 4-7 characterized in that the amphiphatic and polar lipid components is a phosphatidylcholine and the non polar component is a monoglyceride, which is composed essentially of 8:0 caprylate and 10:0 caprate.

9. A composition according to any of claims 1-8 characterized in that the heparin or a fragment or a derivative thereof is present in an amount of 1-200% (w/w) with respect to the lipid system, preferably between 4-80% (w/w).

10. A composition according to any of claims 1-9 characterized in that the heparin is low molecular weight fragment with a molecular mass of about 2000-10000 Da.

11. A topical preparation containing the composition according to any of claims 1-10 and suitable excipients.

12. A method for preparing the compositions according to any of claims 1-10 characterized by adding a solution of the heparin or a fragment or a derivative thereof to a stirred mixture of the lipid components, thereafter adding the water containing solvent and treating the resultant mixture mechanically or alternatively mixing the heparin or its fragments or derivatives with the water containing solvent before adding the mixture to the stirred lipid components.

13. Use of a composition according to any of the previous claims for the manufacture of a preparation for oral, buccal, sublingual, rectal or transdermal administration or for administration to mucous membranes.

14. A method for increasing the antithrombotic activity in blood plasma and/or for the lysis of preformed blood clots **characterized by** administering a therapeutically effective amount of the composition according to claims 1-10 and suitable excipients to a patient.

15. A method for prevention and/or treatment of atherosclerosis **characterized by** administering the composition according to any of claims 1-10 and suitable excipients to a patient.

16. A method for treatment of ischemic heart diseases and related vascular disorders **characterized by** administering a therapeutically effective amount of the composition according to claims 1-10 and suitable excipients.

17. A method for treatment of inflammation **characterized by** administering the composition according to any of claims 1-10 and suitable excipients to a patient.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 93/00258

A. CLASSIFICATION OF SUBJECT MATTER		
IPC5: A61K 9/127, A61K 47/44, B01J 13/02 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC5: A61K, B01J		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
SE,DK,FI,NO classes as above		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
MEDLINE, EMBASE, WPI, WPIL, CLAIMS, CA		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO, A1, 9205771 (KABI PHARMACIA AB), 16 April 1992 (16.04.92), see the whole document --	1-9
Y	EP, A2, 0158441 (PHARES PHARMACEUTICAL RESEARCH N.V.), 16 October 1985 (16.10.85), see page 5, line 1 - page 6, line 16; page 8, line 5 - page 9, line 18; page 12, line 18 - line 34 --	1-9
Y	WO, A1, 8707502 (PHARES PHARMACEUTICAL RESEARCH N.V.), 17 December 1987 (17.12.87), see pages 1-4, example 5 and the claims --	1-9
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "B" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "Z" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
22 June 1993		29 -06- 1993
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INTERNATIONAL SEARCH REPORT

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>Dialog Information Services, file 154, Medline 1985-1992/DEC, Dialog accession no. 06045730/5, Medline accession no. 87019730, Kim T.D. et al: "Studies on liposoma-encapsulated heparin", Thromb Res Sep 15 1986, 43 (6) p603-12</p> <p style="text-align: center;">-- -----</p>	1-9

INTERNATIONAL SEARCH REPORT

In. .ational application No.

PCT/SE 93/00258

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 14-17
because they relate to subject matter not required to be searched by this Authority, namely:
Methods for treatment of the human or animal body by surgery or therapy,
as well as diagnostic methods (see PCT Rule 39(iv)).
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such
an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

28/05/93

International application No.

PCT/SE 93/00258

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9205771	16/04/92	AU-A- 8710591 EP-A- 0514506	28/04/92 25/11/92
EP-A2- 0158441	16/10/85	SE-T3- 0158441 DE-A- 3585967 JP-A- 61044808 US-A- 5004611 US-A- 5053217 US-A- 5141674	11/06/92 04/03/86 02/04/91 01/10/91 25/08/92
WO-A1- 8707502	17/12/87	DE-A- 3783039 EP-A,B- 0309464 SE-T3- 0309464	21/01/93 05/04/89

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- (71) Applicant (for all designated States except US): **BIODE-LIVERY SCIENCES INTERNATIONAL, INC.**
[US/US]; 185 South Orange Avenue, Administrative building 4, Newark, NJ 07103 (US).
- (72) Inventors; and
(75) Inventors/Applicants (for US only): **KRAUSE-ELSMORE, Sara, L.** [US/US]; 100 Maple Street, Kearny, NJ 07032 (US). **MANNINO, Raphael, J.** [US/US]; 36 Meadowview Drive, Annandale, NJ 08801 (US).
- (74) Agents: **HANLEY, Elizabeth, A.** et al.; Lahive & Cockfield, LLP, 28 State Street, Boston, MA 02109 (US).
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: **RIGID LIPOSOMAL COCHLEATE AND METHODS OF USE AND MANUFACTURE**

(57) Abstract: Employing liposomes having a high transition temperature at least partially disposed in a matrix, compositions are provided that can be used to deliver one or more cargo moieties, e.g., a drug, a nutrient, an imaging agent and/or non-steroidal anti-inflammatory. The matrix can be a lipid precipitate and/or a cationic bridge. Methods of making and using these compositions preferably cochleates, are also disclosed.

WO 2005/063213 A1

RIGID LIPOSOMAL COCHLEATE AND METHODS OF USE AND MANUFACTURE

Related Applications

This application claims the benefit of U.S. Provisional Application Nos. 60/531,546, filed December 19, 2003 and 60/565,120, filed April 23, 2004, which
5 applications are incorporated herein by this reference.

Technical Field

The invention generally relates to compositions including high transition temperature liposomes at least partially disposed in a matrix.

Background

10 Conventional liposomal suspensions have fluid membranes that can allow encapsulated drugs to leak out over time. Moreover, upon dehydration or exposure to a cationic environment, the lipid bilayers of a liposome can collapse, displacing the aqueous interior of the liposomes, including the encapsulated drug, and exposing the drug to the environment. Consequently, conventional liposomes
15 may inefficiently retain encapsulated drug, exposing the drugs to environmental conditions that can lead to degradation or deactivation.

Summary of the Invention

The present invention provides stable liposome compositions for the delivery of a variety of cargo moieties (*e.g.*, drugs and/or nutrients). The
20 liposomes are formulated such that they are rigid at ambient temperatures. The rigid liposomes preferably are at least partially disposed in a matrix, such as a cation bridge or lipid precipitate, that can serve to further stabilize the composition and/or carry additional cargo moieties.

One advantage of such liposome compositions is that cargo moieties
25 associated with the rigid liposomes of the invention (*e.g.*, residing within the liposomal aqueous interior and/or associated with the lipid bilayer) cannot readily

dissociate from the liposome. The invention is particularly advantageous when a cargo moiety is disposed in the aqueous interior of the liposomes, which typically would be displaced upon dehydration and/or addition of cation. When the rigid liposomes of the invention are exposed to conditions in which conventional
5 liposomes would collapse and/or precipitate, the rigid liposomes do not collapse and the associated cargo moieties are retained and protected.

Another advantage of the compositions of the present invention is that they can be formulated so that the transition temperature is at or below the body temperature of an animal such that the rigid liposomes will become fluid after
10 ingestion, facilitating delivery of cargo moieties to the animal. Another advantage of the current invention is that a matrix can be used to deliver cargo moieties as well. The cargo moieties can exist, *e.g.*, in a cationic bridge or a lipid bilayer of a precipitate.

Brief Description of the Drawings

15 *Figure 1A* is an image of crystalline distearoyl phosphatidylserine (DSPS) upon direct addition to water and Rhodamine labeled Dextran (Rho-Dextran) at a temperature below its transition temperature (68°C).

Figure 1B is an image of fluid DSPS liposomes formed upon heating and periodic vortexing of the crystalline DSPS depicted in *Figure 1A* above the
20 transition temperature.

Figure 1C is an image of an exemplary composition of the invention including the rigid DSPS liposomes of *Figure 1B*, at a temperature below its transition temperature and disposed in a calcium matrix.

Figure 1D is an image of the composition of *Figure 1C* after the addition
25 of EDTA at a temperature below the lipid bilayer transition temperature. The EDTA did not appear to induce an observable change in the structure.

Figure 2A is an image of rigid DSPS Rho-Dextran liposomes disposed in a matrix formed from DOPS liposomes precipitated with calcium. The image was captured with phase contrast microscopy. The image indicates that the Rho-
30 Dextran is maintained within the rigid liposomes.

Figure 2B is an image of the composition of *Figure 2A* captured by employing both fluorescence and phase contrast microscopy.

Figure 2C is an image of the composition of Figure 2A captured by employing fluorescence microscopy.

Detailed Description of the Invention

In conventional liposomes, fluidity of the lipid bilayer can contribute to
5 leaking or loss of cargo moieties from their interior and/or the bilayer. Membrane fluidity, however, is a key factor in the successful fusion of liposomes and lipid precipitates (e.g., cochleates) with membranes. A new composition has been discovered that provides stability at ambient temperatures and fluidity at high transition temperatures (e.g., the body temperatures of animals such as humans).
10 Such a composition provides a delivery vehicle stable at temperatures normally experienced during storage and shipping but that is capable of delivery upon administration to a subject.

The lipid structures of the present invention are particularly advantageous because they are capable of retaining cargo moieties which can leak out of
15 conventional liposomes and potentially, in some instances, cochleates, e.g., hydrophilic cargo moieties. Such cargo moieties are retained in the lipid structure until it is heated to above the lipid bilayer transition temperature because of the rigidity of the system at temperatures lower than the transition temperature.

In one aspect, the composition includes a plurality of liposomes having a
20 high transition temperature, i.e., "rigid liposomes," at least partially disposed in a matrix. In one embodiment, the matrix is a cation bridge between at least a portion of the plurality of rigid liposomes. Surprisingly, in these embodiments, even addition of a chelating agent, which typically would open up a lipid precipitate, does not induce a change in the structure. When heated above the
25 transition temperature in the presence of chelating agent, however, the structure opens up and the liposomes are capable of fusion.

Additionally or alternatively, the rigid liposomes can be disposed in a matrix including lipid precipitate including a charged lipid and a multivalent counter ion, e.g., a cochleate formed from multivalent cation and liposomes
30 including negatively charged lipid. The lipid precipitate also can include additional cargo moieties and/or can have, but is not limited to a high transition temperature.

In order to more clearly and concisely describe the subject matter of the claims, the following definitions are intended to provide guidance as to the meaning of specific terms used in the following written description, examples and appended claims.

5 A "liposome" is a structure composed of at least one lipid bilayer disposed about a typically aqueous volume. Liposomes can be formed from a wide variety of components well known in the art. For example, liposomes may include lipids such as phospholipids, and also may include other lipids such as cholesterol.

10 The term "transition temperature" refers to the temperature at which a lipid bilayer begins to transition from a rigid to a fluid state. The term "high transition temperature" refers to a lipid bilayer transition temperature that is higher than at least about 20°C. Liposomes with lipid bilayers having a high transition temperature are referred to herein as "rigid liposomes" as they are less fluid at ambient temperatures than conventional liposomes that have lower transition
15 temperature lipid bilayers.

The term "precipitate," as used herein, refers to precipitates of charged lipid and multivalent counter ion. A precipitate can be a cochleate if it takes on an alternating cationic and lipid bilayer structure. However, a precipitate can have alternative structures as well.

20 As used herein, the term "cochleate" refers to lipid precipitates that include alternating lipid bilayer and counter-ion sheets, stacked and/or rolled up with little or no internal aqueous space. The term "enocochleated" means associated with a cochleate structure, *e.g.* by incorporation into the cationic sheet, and/or inclusion in the lipid bilayer. Methods of making and using cochleates are described, *e.g.*, in
25 U.S. Patent Nos. 5,994,318 and 6,153,217, which are incorporated in their entirety by this reference. Accordingly, an enocochleated rigid liposome would include at least one rigid liposome disposed at least partially in a cochleate matrix.

As used herein, the term "multivalent cation" refers to a divalent cation or higher valency cation, or any compound that has at least two positive charges,
30 including mineral cations such as calcium, barium, zinc, iron and magnesium and other elements capable of forming ions or other structures having multiple positive charges capable of chelating and bridging negatively charged lipids. Additionally or alternatively, the multivalent cation can include other multivalent cationic

compounds, *e.g.*, cationic cargo moieties. The term "divalent metal cation," as used herein, refers to a metal having two positive charges.

The term "matrix" refers to a component that binds at least two liposomes. The matrix can be, *e.g.*, an ionic bridge between two liposomes that contain charged lipid, *e.g.*, a multivalent cationic bridge between negatively charged lipid in the high transition temperature liposomes. Additionally or alternatively, the matrix can be a lipid-cation precipitate, for example a cochleate component and/or a precipitate of high transition temperature liposomes and a multivalent cation, optionally incorporating liposomes that do not have a high transition temperature.

A "cargo moiety" is a moiety associated with the compositions of the invention, and generally does not refer to the lipid employed to form the liposomes or precipitates. Cargo moieties also include any compounds having a property of biological interest, *e.g.*, ones that have a role in the life processes of a living organism. A cargo moiety may be organic or inorganic, a monomer or a polymer, endogenous to a host organism or not, naturally occurring or synthesized *in vitro* and the like. In some embodiments, the cargo moiety does not refer to the counterion employed to form the matrix.

In one aspect, the present invention provides a composition that generally includes a plurality of liposomes having a high lipid bilayer transition temperature at least partially disposed in a matrix. These "rigid liposomes" are less fluid at ambient temperatures than conventional liposomes that have lower lipid bilayer transition temperatures.

The fluidity of a lipid bilayer depends on both its composition and temperature, as is readily demonstrated in studies of synthetic bilayers. A synthetic bilayer made from a single type of phospholipid transitions from a rigid crystalline state to a liquid state at a characteristic transition point or lipid bilayer transition temperature. When there is more than one type of lipid and/or additional components present in the lipid bilayer, the lipid bilayer transition can occur over a temperature range, gradually becoming less fluid as the temperature decreases. The lipid bilayer transition temperature is the temperature at which the transition from solid to liquid begins.

A practitioner can readily determine the transition temperature of lipids and lipid mixtures from, *e.g.*, product literature, lipid databases, and routine experimentation. For example, lipids having a high transition temperature can be identified readily employing on-line databases, such as the lipid databases
5 maintained by Ohio State University (<http://www.lipidat.chemistry.ohio-state.edu>) and Avanti Polar Lipids (www.avantilipids.com).

Additionally, the transition temperature of a lipid bilayer readily can be determined by heating a lipid or lipid mixture from below the transition temperature while observing the transition under a microscope. The lipid bilayer
10 transition temperature can be readily adjusted by employing other lipids or cholesterol. While transition temperatures of a large number of lipids are readily available, a general knowledge of the affect of structure on transition temperatures can also be employed to choose lipids. Generally, the shorter the hydrocarbon chain/or and the more double bonds, the lower the transition temperature. A
15 shorter chain length reduces the tendency of the hydrocarbon tails to interact with one another, and *cis*-double bonds produce kinks in the hydrocarbon chains that make them more difficult to pack together, so that the membrane remains fluid at lower temperatures. Moreover, the head group of a fatty acid can affect the transition temperature (*e.g.*, a choline head group lowers the transition temperature
20 relative to an ethanolamine head group). Additional substituents, *e.g.*, cholesterol, can broaden the range of transition because they inhibit regularity in the bilayer. Employing the teachings contained herein, the skilled practitioner can readily manufacture the compositions of the invention using no more than routine experimentation.

25 Accordingly, in some embodiments, the liposomes include one or more saturated phospholipids and/or phospholipids having a chain length of 16 to 24 carbons.

In one embodiment, the transition temperature of the lipid bilayer is at least about 30°C. In one embodiment, the transition temperature is at least about 45°C
30 and in another embodiment, the transition temperature is at least about 60°C. In certain embodiments, the transition temperature is below about 100°C. In some embodiments, the lipid bilayer transition temperature is between about 25°C and 95°C. In other embodiments, the lipid bilayer transition temperature is between

about 30°C and 85°C. In yet other embodiments, the lipid bilayer transition temperature is between about 35°C and 75°C. In one embodiment, the transition temperature of the lipid bilayer is lower than body temperature, *e.g.*, below about 40°C.

5 In other embodiments, the transition temperature is at least about 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40 ... 100°C. All individual values and ranges within this range are considered to be embodiments of the invention and can be chosen based on the specific manufacturing, storage, and/or delivery conditions to which the compositions will
10 be exposed. For example, the transition temperature can be chosen to be higher than storage conditions but lower than or about equal to the body temperature of an animal to which the compositions will be administered.

In another embodiment, the transition temperature of the lipid bilayer is higher than a human or other animal body temperature. One advantage of such a
15 rigid lipid structure is that the structure may not allow fusion such that, *e.g.*, imaging agents are not released but retained within the rigid lipid structure.

In one embodiment, the liposomes include a majority of charged lipid. In certain embodiments, the liposomes of the invention include at least about 75%, at least about 80%, at least about 90%, or at least about 95%, charged lipid. In some
20 embodiments, the liposomes include one or more phospholipids. In one embodiment, the phospholipid is a charged phospholipid. The liposomes can include negatively charged, positively charged and/or neutral phospholipids. In certain embodiments, the lipid is a negatively charged phospholipid.

In one embodiment, the liposomes include at least one phospholipid
25 selected from the group consisting of: distearoyl phosphatidylserine (DSPS), distearoyl phosphatidylcholine (DSPC), dipalmitoyl phosphatidic acid (DPPA), dipalmitoyl phosphatidylcholine (DPPC), dipalmitoyl phosphatidylethanolamine (DPPE), dipalmitoyl phosphatidylserine (DPPS), dipalmitoyl phosphatidylglycerol (DPPG), dimyristoyl phosphatidylethanolamine (DMPE), dioleoyl phosphatidic acid (DOPA), and dimyristoyl phosphatidylserine (DMPS).
30

In certain embodiments, the rigid liposomes are disposed, at least in part, in a matrix. In one embodiment, the matrix includes an ionic bridge between the rigid liposomes. The ionic bridge can be a multivalent ion that having a charge

opposite that of some of the lipids employed to form the rigid liposomes. For example, where the plurality of liposomes includes a negatively charged lipid, the matrix can be a cationic bridge between a multivalent cation and the negatively charged lipid. Similarly, where the plurality of liposomes includes a positively charged lipid, the matrix can be an anionic bridge between a multivalent anion and the positively charged lipid.

In some embodiments, more than one, *e.g.*, two or three or more, populations or formulations of rigid liposomes may be disposed in a matrix. For example, a matrix may incorporate rigid liposomes which include a first lipid and a first cargo moiety and rigid liposomes which include a second lipid and a second cargo moiety. Alternatively, a matrix may incorporate rigid liposomes which include a first lipid and a first cargo moiety and rigid liposomes which include the first lipid and a second cargo moiety. In yet another example, a matrix may incorporate rigid liposomes which include a first lipid and a first cargo moiety and rigid liposomes which include a second lipid and the first cargo moiety. Such formulations may be advantageous, *e.g.*, for coadministration of two or more cargo moieties or for varying release profiles.

Additionally or alternatively, the matrix can include a precipitate that generally includes a multivalent ion and a charged lipid (*e.g.*, a multivalent cation and a negatively charged lipid). The matrix can be a cochleate or other structure. The lipid can include, but is not limited to, high transition temperature lipids. The precipitate can include an ionic bridge between the precipitate and the rigid liposomes. Alternatively, the matrix can be disposed about the liposome without a chemical, ionic or other bridge or bond.

In another aspect, the present invention includes methods for forming the compositions of the present invention. The method generally includes forming a matrix at least partially about a plurality of liposomes having a high transition temperature. In one embodiment, the plurality of liposomes includes an anionic lipid and the matrix comprises a multivalent cation. The matrix can include a cationic bridge formed by the multivalent cation between the liposomes. Additionally or alternatively, the matrix can include a precipitate of the multivalent cation and a second plurality of liposomes. In a preferred

embodiment, the precipitate is a cochleate that includes the multivalent cation and a second plurality of liposomes

In one embodiment, a matrix can be formed by introducing at least one multivalent ion (*e.g.*, calcium) to the rigid liposomes. In specific embodiments, the multivalent ion is cationic and the liposomes include negatively charged lipids. A unique and surprising structure has been observed to form upon addition of multivalent cation to rigid liposomes including negatively charged lipid. The multivalent ion forms an ionic bridge between the rigid liposomes. In the examples and figures disclosed herein, the structure appears under a microscope as a grape-like cluster of liposomes encrusted or otherwise held together by the cation. Also surprising about this structure, is that the addition of a chelating agent (*e.g.*, EDTA) does not open up the matrix. Without wishing to be bound by any particular theory, it is believed that the bridge between the multivalent ion and the high-transition temperature lipids in the liposomes is "locked" because the lipid does not allow the chelating agent to access the multivalent ion.

In another embodiment, the matrix is formed by introducing or forming a plurality of liposomes that have a lower lipid bilayer transition temperature and precipitating these liposomes with a counter ion. Such liposomes and resulting precipitates can include lipids, lipid mixtures, cargo moieties, cations and other components described, *e.g.*, in WO 2004091579, published October 28, 2004 and entitled "Novel Encochleation Methods, Cochleates, and Methods of Use" and in WO 2004091579, published October 28, 2004 and entitled "Cochleate Compositions Directed Against the Expression of Proteins," both of which are incorporated herein by this reference in their entireties. The methods described in WO 2004091579 and WO 2004091579 can also be readily employed in connection with the present invention. The precipitate optionally can be formed in the presence of additional cargo moieties such that the cargo moieties are incorporated into the matrix. These cargo moieties can be the same or different from the cargo moieties associated with the rigid liposomes. The ion can also form an ionic bridge between the precipitate and the liposomes.

For example, one or more multivalent cations can be introduced to suspension including a plurality of rigid liposomes and a plurality of liposomes having a lower lipid bilayer transition temperature, both including negatively

charged lipid. In certain embodiments, the multivalent cation precipitates with calcium about the liposomes. In other embodiments, the multivalent cation also forms a bridge with the negatively charged lipid in the rigid liposomes.

In still other embodiments, a matrix may be formed by introducing a plurality of liposomes which include a negatively charged lipid to a plurality of liposomes that have a high transition temperature include little or no negatively charged lipid, and precipitating the negatively charged lipid with a counter ion. Without wishing to be bound by any particular theory, it is believed that the rigid liposomes would not interact with the counter ion, but may be at least partially trapped within the matrix.

In another embodiment, the matrix includes matrix portions that are precipitates and/or cochleates, and portions that are cationic bridges between rigid liposomes.

In one embodiment, the matrix is disposed completely or substantially about the plurality of liposomes having a high transition temperature. In another embodiment, the matrix is only partially disposed about the liposomes. In some embodiments, the matrix is formed in several steps, *e.g.*, forming a first precipitate with a cargo moiety, and then forming a second precipitate with another cargo moiety. Multiple variations of this method can be employed and are well within the scope of this invention. For example, the rigid liposomes including a first cargo moiety can be at least partially disposed in a matrix that is a cationic bridge and includes a second cargo moiety, subsequently a further precipitate can be formed with a plurality of liposomes, cation and a third moiety. Another example is the use of at least two different types of liposomes for forming the precipitate with different lipid content and/or different cargo moieties associated with each.

In one embodiment the method includes the step of forming of a plurality of liposomes with a high lipid bilayer transition temperature. The liposomes can be formed, *e.g.*, by heating an aqueous mixture of lipids above the lipid bilayer transition temperature. Alternatively, the lipid can be added to an aqueous media at a temperature above the lipid bilayer transition temperature. In certain embodiments, the mixture is mixed, sonicated, or vortexed to hasten the formation of liposomes.

In one embodiment, the method includes introducing at least one cargo moiety for association with the rigid liposomes. Cargo moieties can be associated with the structures in a variety of ways. For example, the cargo moieties can be disposed in the interior of the rigid liposomes and/or associated with the rigid liposome bilayer. Generally, hydrophilic moieties will be entrapped in the liposomal aqueous interior, and moieties having both hydrophilic and hydrophobic portions will associate with the lipid bilayer. Hydrophobic moieties can associate with the lipid bilayer and/or can exist in emulsion in the aqueous interior of the liposomes.

The rigid liposomes can include rigid liposomes with different lipid mixtures and/or different cargo moieties. This can be achieved by a variety of methods, *e.g.*, by mixing the different types of rigid liposomes together after formation and/or solidification. This also can be achieved, *e.g.*, by forming a first plurality of liposomes having a first lipid bilayer transition temperature, cooling below the first transition temperature, forming a second plurality of rigid liposomes having a second lipid bilayer transition temperature below the first transition temperature, and cooling below the second transition temperature. The same or different cargo moieties can be introduced for association with each plurality of liposomes.

In one embodiment, liposomes with a high lipid bilayer transition temperature are formed primarily or exclusively from DSPS, which has a transition temperature of about 68°C. When heated above its transition temperature in aqueous solution including a desired cargo moiety, liposomes form than include an aqueous solution including cargo moiety. When the liposomes are cooled below the lipid bilayer transition temperature, *e.g.*, room temperature, the liposomes become rigid.

The liposomes can optionally then be washed or otherwise processed to remove all or substantially all of the cargo moiety that is not associated with the rigid liposomes. Such a step can be desirable, *e.g.*, when the cargo moiety is toxic or inflammatory and/or so that it can be recycled into another batch of rigid liposomes. Additionally or alternatively, the liposomes can be washed after the formation of the matrix. These compositions can be particularly advantageous, *e.g.*, in oral preparations where the cargo moiety is toxic or otherwise harmful to

the GI tract and/or the cargo moiety is susceptible to degradation in the GI tract, because the cargo moiety will not be released from the composition until after it passes into the bloodstream. Accordingly, the GI tract is protected from the cargo moiety and the cargo moiety is protected from the GI tract.

5 Additionally or alternatively, cargo moieties can be associated with the matrix. For example they can be disposed in a cationic bridge (*e.g.*, a calcium cation bridge formed between rigid liposomes), or in a lipid-cation precipitate (*e.g.*, in a cochleate matrix disposed at least partially about a plurality of rigid liposomes). The structure can have more than one cargo moiety incorporated in
10 more than one manner. For example, one type of cargo moiety can be disposed with a cochleate matrix for more immediate delivery, and a second type of cargo moiety can be disposed in the rigid liposome structure for later delivery.

 Additionally or alternatively, cargo moieties can be disposed in conventional liposomes included (*e.g.*, in an emulsion including rigid liposomes)
15 in the compositions of the present invention. While typically, such delivery is less efficient, it may be useful in some circumstances (*e.g.*, when loss of cargo moiety in the conventional liposomes is desired and/or acceptable). By way of example, a composition of the invention might include a drug that is harmful to the stomach that is entrapped within rigid liposomes and a second drug or other agent that
20 protects the stomach disposed in conventional liposomes (*i.e.*, liposomes that do not have a high transition temperature) and/or a cochleate matrix. Numerous other variations readily can be envisioned by a practitioner based on the present specification.

 Classes of cargo moieties include but are not limited to vitamins, minerals,
25 nutrients, micronutrients, amino acids, toxins, microbicides, microbistats, co-factors, enzymes, polypeptides, polypeptide aggregates, polynucleotides, lipids, carbohydrates, nucleotides, starches, pigments, fatty acids, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, flavorings, essential oils, extracts, hormones, cytokines, viruses, organelles, steroids and other multi-ring
30 structures, saccharides, metals, metabolic poisons, antigens, imaging agents, porphyrins, tetrapyrrolic pigments, drugs and the like.

 The cargo moiety can be a diagnostic agent, such as an imaging agent. Imaging agents include nuclear agents and porphyrins. Porphyrins include

tetrapyrrolic agents or pigments. One such tetrapyrrolic agent is Zinc Tetra-Phenyl Porphyrin (ZnTPP), which is a hydrophobic, fluorescent molecule that has high absorption in the visible spectrum (dark purple).

The cargo moiety can be a polynucleotide that is expressed to yield a
5 biologically active polypeptide or polynucleotide. Thus, the polypeptide may serve as an immunogen or, *e.g.*, have enzymatic activity. The polynucleotide may have catalytic activity, *e.g.*, be a ribosome, or may serve as an inhibitor of transcription or translation, *e.g.*, a small interfering RNA (siRNA) or an antisense molecule. The polynucleotide can be an antisense molecule including a modified
10 antisense molecule, such as a morpholino antisense molecule. The polynucleotide can be modified, *e.g.*, it can be synthesized to have a morpholino backbone. If expressed, the polynucleotide preferably includes the necessary regulatory elements, such as a promoter, as known in the art. A specific example of a polypeptide is insulin.

15 The cargo moiety can be an organic molecule that is hydrophobic in aqueous media. The cargo moiety can also be a water-soluble monovalent or polyvalent cationic molecule, anionic, or net neutral at physiological pH.

The cargo moiety can be a drug, such as, a protein, a small peptide, a bioactive polynucleotide, an antibiotic, an antiviral, an anesthetic, an
20 antipsychotic, an anti-infectious, an antifungal, an anticancer, an immunosuppressant, an immunostimulant, a steroidal anti-inflammatory, a non-steroidal anti-inflammatory, an antioxidant, an antidepressant which can be synthetically or naturally derived, a substance which supports or enhances mental function or inhibits mental deterioration, an anticonvulsant, an HIV protease
25 inhibitor, a non-nucleophilic reverse transcriptase inhibitor, a cytokine, a tranquilizer, a mucolytic agent, a dilator, a vasoconstrictor, a decongestant, a leukotriene inhibitor, an anti-cholinergic, an anti-histamine, a cholesterol lipid metabolism modulating agent, or a vasodilatory agent. The drug can also be any over the counter (non-prescription) medication. Examples include Amphotericin
30 B, acyclovir, adriamycin, carbamazepine, ivermectin, melphalen, nifedipine, indomethacin, curcumin, aspirin, ibuprofen, naproxen, acetaminophen, rofecoxib, diclofenac, ketoprofen, meloxicam, nabumetone, estrogens, testosterone, steroids, phenytoin, ergotamines, cannabinoids, rapamycin, propanadid, propofol,

alphadione, echinomycin, miconazole, miconazole nitrate, ketoconazole,
 itraconazole, fluconazole, griseofulvin, clotrimazole, econazole, terconazole,
 butoconazole, oxiconazole, sulconazole, saperconazole, voriconazole, ciclopirox
 olamine, haloprogin, tolnaftate, naftifine, terbinafine hydrochloride, morpholines,
 5 flucytosine, natamycin, butenafine, undecylenic acid, Whitefield's ointment,
 propionic acid, and caprylic acid, clioquinol, selenium sulfide, teniposide,
 hexamethylmelamine, taxol, taxotere, 18-hydroxydeoxycorticosterone,
 prednisolone, dexamethazone, cortisone, hydrocortisone, piroxicam, diazepam,
 verapamil, vancomycin, tobramycin, teicoplanin, bleomycin, peptidoglycan,
 10 ristocetin, sialoglycoproteins, orienticin, avaporcin, hevecardin, galacardin,
 actinoidin, gentamycin, netilmicin, amikacin, kanamycin A, kanamycin B,
 neomycin, paromomycin, neamine, streptomycin, dihydrostreptomycin,
 apramycin, ribostamycin, spectinomycin, caspofungin, echinocandin B, aculeacin
 A, micafungin, anidulafungin, cilofungin, pneumocandin, geldanamycin, nystatin,
 15 rifampin, tyrphostin, a glucan synthesis inhibitor, vitamin A acid, mesalamine,
 risedronate, nitrofurantoin, dantrolene, etidronate, nicotine, amitriptyline,
 clomipramine, citalopram, dothepin, doxepin, fluoxetine, imipramine,
 lofepramine, mirtazapine, nortriptyline, paroxetine, reboxetine, sertraline,
 trazodone, venlafaxine, dopamine, St. John's wort, phosphatidylserine,
 20 phosphatidic acid, amastatin, antipain, bestatin, benzamidine, chymostatin, 3,4-
 dichloroisocoumarin, elastatinal, leupeptin, pepstatin, 1,10-phenanthroline,
 phosphoramidon, ethosuximide, ethotoin, felbamate, fosphenytoin, lamotrigine,
 levitracetam, mephentoin, methsuximide, oxcarbazepine, phenobarbital,
 phensuximide, primidone, topirimate, trimethadione, zonisamide, saquinavir,
 25 ritonavir, indinavir, nelfinavir, and amprenavir.

An antifungal drug can be a polyene macrolide, tetraene macrolide,
 pentaenic macrolide, fluorinated pyrimidine, imidazole, azole, triazole,
 halogenated phenolic ether, thiocarbamate, allylamine, sterol inhibitor, and an
 agent that interpolates fungal cell wall components.

30 The drug can be a polypeptide such as cyclosporin, Angiotensin I, II and
 III, enkephalins and their analogs, ACTH, anti-inflammatory peptides I, II, III,
 bradykinin, calcitonin, b-endorphin, dinorphin, leucokinin, leutinizing hormone

releasing hormone (LHRH), insulin, neurokinins, somatostatin, substance P, thyroid releasing hormone (TRH) and vasopressin.

The drug can be an antigen, but is not limited to a protein antigen. The antigen can also be a carbohydrate or DNA. Examples of antigenic proteins
5 include membrane proteins, carbohydrates, envelope glycoproteins from viruses, animal cell proteins, plant cell proteins, bacterial proteins, and parasitic proteins.

The cargo moiety can be a nutrient, including, but not limited to lycopene, micronutrients such as phytochemicals or zoochemicals, vitamins, minerals, fatty acids, amino acids, fish oils, fish oil extracts, and saccharides, herbal products,
10 essential oils, or flavor agents. Specific examples include Vitamins A, B, B1, B2, B3, B12, B6, B-complex, C, D, E, and K, vitamin precursors, carotenoids, and beta-carotene, resveratrol, biotin, choline, inositol, ginkgo, lutein, zeaxanthine, quercetin, silibinin, perillyl alcohol, genistein, sulfurophane, essential fatty acids, including eicosapentanoic acid (EPA), gamma-3, omega-3, gamma-6, and omega-
15 6 fatty acids, herbs, spices, and iron. Minerals include, but are not limited to boron, chromium, colloidal minerals, colloidal silver, copper, manganese, potassium, selenium, vanadium, vanadyl sulfate, calcium, magnesium, barium, iron and zinc.

As used herein, "micronutrient" is a nutrient that the body must obtain
20 from outside sources. Generally micronutrients are essential to the body in small amounts.

The cargo moiety can be a saccharide or sweetener, *e.g.*, saccharine, isomalt, maltodextrine, aspartame, glucose, maltose, dextrose, fructose and sucrose. Flavor agents include oils, essential oils, or extracts, including but not
25 limited to oils and extracts of cinnamon, vanilla, almond, peppermint, spearmint, chamomile, geranium, ginger, grapefruit, hyssop, jasmine, lavender, lemon, lemongrass, marjoram, lime, nutmeg, orange, rosemary, sage, rose, thyme, anise, basil, and black pepper, tea or tea extracts, an herb, a citrus, a spice or a seed.

In yet another aspect, the present invention provides for both prophylactic
30 and therapeutic methods of treating a subject at risk of (or susceptible to) a disorder or having a disorder which can be treated with one or more cargo moiety.

"Treatment", or "treating" as used herein, is defined as the application or administration of a therapeutic agent (*e.g.*, antibiotics) to a subject or patient, or

application or administration of a therapeutic agent to an isolated tissue or cell line from a subject or patient, who has a disease or disorder, a symptom of disease or disorder or a predisposition toward a disease or disorder, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve or affect the disease or disorder, the symptoms of the disease or disorder, or the predisposition toward disease. "Treated," as used herein, refers to the disease or disorder being cured, healed, alleviated, relieved, altered, remedied, ameliorated improved or affected. For example, certain methods of treatment of the instant invention provide for administration of anti-inflammatory compositions, such that inflammation is lessened or alleviated.

The terms "cure," "heal," "alleviate," "relieve," "alter," "remedy," "ameliorate," "improve" and "affect" are evaluated in terms of a suitable or appropriate control. A "suitable control" or "appropriate control" is any control or standard familiar to one of ordinary skill in the art useful for comparison purposes. In one embodiment, a "suitable control" or "appropriate control" is a value, level, feature, characteristic, property, etc. determined prior to administration of a cargo moiety composition, as described herein. For example, the number of colony forming units can be determined prior to administering an antifungal composition of the invention to a host. In another embodiment, a "suitable control" or "appropriate control" is a value, level, feature, characteristic, property, etc. determined in a subject, *e.g.*, a control or normal subject exhibiting, for example, normal traits. In yet another embodiment, a "suitable control" or "appropriate control" is a predefined value, level, feature, characteristic, property, etc.

The methods of the present invention include methods of administering a cargo moiety to a host, wherein the cargo moiety is associated with a composition of the invention. The compositions of the present invention may be administered orally, nasally, topically, intravenously, transdermally, buccally, sublingually, rectally, vaginally or parenterally.

The present invention provides a method for treating a subject that would benefit from administration of a composition of the present invention. Any therapeutic indication that would benefit from a cargo moiety, *e.g.*, a drug or nutrient, can be treated by the methods of the invention. Accordingly, the present invention provides methods of treating a subject at risk for or having a disease or

disorder which can be treated with, for example, a protein, a small peptide, a bioactive polynucleotide, an antibiotic, an antiviral, an anesthetic, antipsychotic, an anti-infectious, an antifungal, an anticancer, an immunosuppressant, an immunostimulant, a steroidal anti-inflammatory, a non-steroidal anti-inflammatory, an antioxidant, an antidepressant which can be synthetically or naturally derived, a substance which supports or enhances mental function or inhibits mental deterioration, an anticonvulsant, an HIV protease inhibitor, a non-nucleophilic reverse transcriptase inhibitor, a cytokine, a tranquilizer, a mucolytic agent, a dilator, a vasoconstrictor, a decongestant, a leukotriene inhibitor, an anti-cholinergic, an anti-histamine, a cholesterol lipid metabolism modulating agent or a vasodilatory agent. The method includes the step of administering to the subject a composition of the invention, such that the disease or disorder is treated.

The disease or disorder can be, *e.g.*, inflammation, pain, infection, fungal infection, bacterial infection, viral infection, parasitic disorders, an immune disorder, genetic disorders, degenerative disorders, cancer, proliferative disorders, obesity, depression, hair loss, impotence, hypertension, hypotension, dementia, senile dementia, or malnutrition, acute and chronic leukemia and lymphoma, sarcoma, adenoma, carcinomas, epithelial cancers, small cell lung cancer, non-small cell lung cancer, prostate cancer, breast cancer, pancreatic cancer, hepatocellular carcinoma, renal cell carcinoma, biliary cancer, colorectal cancer, ovarian cancer, uterine cancer, melanoma, cervical cancer, testicular cancer, esophageal cancer, gastric cancer, mesothelioma, glioma, glioblastoma, pituitary adenomas, schizophrenia, obsessive compulsive disorder (OCD), bipolar disorder, Alzheimer's disease, Parkinson's disease, cell proliferative disorders, blood coagulation disorders, Dysfibrinogenaemia and hemophilia (A and B), autoimmune disorders, *e.g.*, systemic lupus erythematosus, multiple sclerosis, myasthenia gravis, autoimmune hemolytic anemia, autoimmune thrombocytopenia, Grave's disease, allogenic transplant rejection, ankylosing spondylitis, psoriasis, scleroderma, uveitis, eczema, dermatological disorders, hyperlipidemia, hyperglycemia, and/or hypercholesterolemia.

The compositions and methods of the instant invention may also be used to promote greater health or quality of life, for example limit cholesterol uptake or regulate lipid metabolism, weight gain, hunger, aging, or growth. Cosmetic

effects such as wrinkle reduction, hair growth, pigmentation, or dermatologic disorders may also be treated. The compositions and methods may also treat hereditary disease such as cystic fibrosis or muscular dystrophy.

- The compositions and methods of the instant invention may be used to
- 5 treat a variety of inflammations, including headache, arthritis, rheumatoid arthritis, osteoarthritis, atherosclerosis, acute gout, acute or chronic soft tissue damage associated with, *e.g.*, a sports injury, tennis elbow, bursitis, tendonitis, acute or chronic back pain, such as a herniated disc, carpal tunnel syndrome, glomerulonephritis, carditis, ulcerative colitis, asthma, sepsis, and plantar fasciitis.
- 10 The compositions and methods of the invention may also be used to relieve pain resulting from surgery or other medical procedure. The compositions and methods of the instant invention may further be used to treat a variety of fungal infections, including candida, *e.g.*, yeast infection, tinea, *e.g.*, Athlete's foot, pityriasis, thrush, cryptococcal meningitis, histoplasmosis, and blastomycosis.
- 15 The compositions and methods of the instant invention may also be used to treat a variety of bacterial infections, including but not limited to moderate to severe lower respiratory tract infections, skin infections, biliary tract infections, bone infections, antibiotic prophylaxis, pseudomembraneous enterocolitis, central nervous system infections (*e.g.*, meningitis and ventriculitis), intra-abdominal
- 20 infections (*e.g.*, peritonitis), pneumonia, septicemia, soft tissue infections, neutropenic sepsis, joint infections, infective endocarditis, and urinary tract infections.

Exemplary bacteria that may be treated with the antibiotic preparation of the present invention include, but are not limited to, *Staphylococcus aureus*,

25 *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Streptococcus Group D*, *Clostridium perfringens*, *Haemophilus influenzae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*.

The compositions and methods of the present invention may be employed for treating a variety of fungal infections, including, but not limited to, asthma,

30 chronic rhinosinusitis, allergic fungal sinusitis, sinus mycetoma, non-invasive fungus induced mucositis, non-invasive fungus induced intestinal mucositis, chronic otitis media, chronic colitis, inflammatory bowel diseases, ulcerative colitis, Crohn's disease, candidemia, intraabdominal abscesses, peritonitis, pleural

space infections, esophageal candidiasis and invasive aspergillosis. Exemplary fungi that can be treated using antifungal compositions of the invention include, without limitation, Absidia, Acinteobacter, Aspergillus flavus, Aspergillus fumigatus, Aspergillus glaucus, Aspergillus nidulans, Aspergillus terreus,

5 Aspergillus versicolor, Alternaria, Basidiobolus, Bipolaris, Candida albicans, Candida glabrata, Candida guilliermondii, Candida krusei, Candida lypolytica, Candida neoformans, Candida parapsilosis, Candida tropicalis, Cladosporium, Conidiobolus, Cunninhamella, Curvularia, Dreschlera, Enterobacter, Exserohilum, Fusarium, Klebsiella, Malbranchia, Paecilomyces, Penicillium,

10 Pseudallescheria, Rhizopus, Schizophyllum, Sporothrix, Acremonium, Arachniotus citrinus, Aurobasidium, Beauveria, Chaetomium, Chrysosporium, Epicoccum, Exophiala jeanselmei, Geotrichum, Oidiodendron, Phoma, Pithomyces, Rhinocladiella, Rhodoturula, Sagrahamala, Scolebasidium, Scopulariopsis, Ustilago, Trichoderma, and Zygomycete.

15 The above methods may be employed in the absence of other treatment, or in combination with other treatments. Such treatments can be started prior to, concurrent with, or after the administration of the compositions of the instant invention.

Accordingly, the methods of the invention can further include the step of

20 administering a second treatment, such as for example, a second treatment for the disease or disorder or to ameliorate side effects of other treatments. Such second treatment can include, *e.g.*, radiation, chemotherapy, transfusion, operations (*e.g.*, excision to remove tumors), and gene therapy. Additionally or alternatively, further treatment can include administration of drugs to further treat the disease or

25 to treat a side effect of the disease or other treatments (*e.g.*, anti-nausea drugs).

With regard to both prophylactic and therapeutic methods of treatment, such treatments may be specifically tailored or modified, based on knowledge obtained from the field of pharmacogenomics. "Pharmacogenomics", as used herein, refers to the application of genomics technologies such as gene sequencing,

30 statistical genetics, and gene expression analysis to drugs in clinical development and on the market. More specifically, the term refers to the study of how a patient's genes determine his or her response to a drug (*e.g.*, a patient's "drug response phenotype", or "drug response genotype"). Thus, another aspect of the invention

provides methods for tailoring an individual's prophylactic or therapeutic treatment according to that individual's drug response genotype. Pharmacogenomics allows a clinician or physician to target prophylactic or therapeutic treatments to patients who will most benefit from the treatment and to
5 avoid treatment of patients who will experience toxic drug-related side effects.

The language "therapeutically effective amount" is that amount necessary or sufficient to produce the desired physiologic response. The effective amount may vary depending on such factors as the size and weight of the subject, or the particular compound. The effective amount may be determined through
10 consideration of the toxicity and therapeutic efficacy of the compounds by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it may be
15 expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to unaffected cells and, thereby, reduce side effects.

20 Subjects at risk for a disease or condition which can be prophylactically treated with the agents mentioned herein can be identified by, *e.g.*, any or a combination of diagnostic or prognostic assays known to those skilled in the art. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the disease or disorder, such that the disease or
25 disorder is prevented or, alternatively, delayed in its progression.

Another aspect of the invention pertains to methods of administering compositions of the invention for therapeutic purposes. In one embodiment, the present invention provides a method for treating a subject that would benefit from administration of a composition of the present invention. Any therapeutic
30 indication that would benefit from administration of a composition of the invention can be treated by the methods of the invention. The present invention provides methods of treating a subject at risk for or having a disease or disorder that can be treated with one or more cargo moieties. The method includes the step

of administering to the subject a composition of the invention, such that the disease or disorder is prevented, ameliorated, terminated or delayed in its progression. The disease or disorder can be any of the diseases or disorders discussed herein.

5 The invention also pertains to uses of the compositions of the invention for prophylactic and therapeutic treatments as described *infra*. Accordingly, the compounds of the present invention can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the compositions of the invention and a pharmaceutically acceptable carrier. As
10 used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art.

15 Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants may also be present in the compositions.

 Examples of pharmaceutically acceptable antioxidants, which may also be
20 present in formulations of therapeutic compounds of the invention, include water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and
25 the like; and metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

 Furthermore, the present invention can further include one or more additional agents, including water, antimicrobial agents, plasticizing agents, flavoring agents, surfactants, stabilizing agents, emulsifying agents, thickening
30 agents, binding agents, coloring agents, sweeteners, fragrances, and the like.

 Suitable antimicrobial agents include triclosan, cetyl pyridium chloride, domiphen bromide, quaternary ammonium salts, zinc compounds, sanguinarine,

fluorides, alexidine, octonidine, EDTA, and essential oils such as thymol, methyl salicylate, menthol and eucalyptol.

Suitable plasticizing agents include, for example, polyols such as sugars, sugar alcohols, or polyethylene glycols (PEGs), urea, glycol, propylene glycol, triethyl citrate, dibutyl or dimethyl phthalate, monoacetin, diacetin or triacetin.

Suitable surfactants include pluronic acid, sodium lauryl sulfate, mono and diglycerides of fatty acids and polyoxyethylene sorbitol esters, such as, Atmos 300 and Polysorbate 80. Suitable stabilizing agents include xanthan gum, locust bean gum, guar gum, and carrageenan. Suitable emulsifying agents include triethanolamine stearate, quaternary ammonium compounds, acacia, gelatin, lecithin, bentonite, veegum, and the like. Suitable thickening agents include methylcellulose, carboxyl methylcellulose, and the like. Suitable binding agents include starch.

Suitable sweeteners that can be included are those well known in the art, including both natural and artificial sweeteners. Suitable sweeteners include water-soluble sweetening agents such as monosaccharides, disaccharides and polysaccharides; water-soluble artificial sweeteners such as soluble saccharin salts, cyclamate salts, or the free acid form of saccharin, and the like; dipeptide based sweeteners, such as L-aspartic acid derived sweeteners; water-soluble sweeteners derived from naturally occurring water-soluble sweeteners, such as a chlorinated derivative of ordinary sugar (sucrose), known, under the product description of sucralose; and protein based sweeteners such as thaumatococcus danielli (Thaumatococcus I and II).

In general, an effective amount of auxiliary sweetener is utilized to provide the level of sweetness desired for a particular composition, and this amount will vary with the sweetener selected. This amount will normally be 0.01% to about 10% by weight of the composition when using an easily extractable sweetener.

The flavorings that can be used include those known to the skilled artisan, such as natural and artificial flavors. These flavorings may be chosen from synthetic flavor oils and flavoring aromatics, and/or oils, oleo resins and extracts derived from plants, leaves, flowers, fruits and so forth, and combinations thereof. Representative flavor oils include: spearmint oil, cinnamon oil, peppermint oil, clove oil, bay oil, thyme oil, cedar leaf oil, oil of nutmeg, oil of sage, and oil of

bitter almonds. Also useful are artificial, natural or synthetic fruit flavors such as vanilla, chocolate, coffee, cocoa and citrus oil, and fruit essences. These flavorings can be used individually or in admixture. Flavorings such as aldehydes and esters including cinnamyl acetate, cinnamaldehyde, citral, diethylacetal, dihydrocarvyl acetate, eugenyl formate, p-methylanisole, and so forth may also be used.
5 Generally, any flavoring or food additive, such as those described in Chemicals Used in Food Processing, publication 1274 by the National Academy of Sciences, pages 63-258, may be used.

10 The amount of flavoring employed is normally a matter of preference subject to such factors as flavor type, individual flavor, and strength desired. Thus, the amount may be varied in order to obtain the result desired in the final product. Such variations are within the capabilities of those skilled in the art without the need for undue experimentation.

15 The compositions of this invention can also contain coloring agents or colorants. The coloring agents are used in amounts effective to produce the desired color. The coloring agents useful in the present invention include pigments such as titanium dioxide, which may be incorporated in amounts of up to about 5 wt %, and preferably less than about 1 wt %. Colorants can also include natural food colors and dyes suitable for food, drug and cosmetic applications. These colorants
20 are known as FD&C dyes and lakes. A full recitation of all FD&C and D&C dyes and their corresponding chemical structures may be found in the Kirk-Othmer Encyclopedia of Chemical Technology, Volume 5, Pages 857-884, which text is accordingly incorporated herein by reference.

25 Formulations of the present invention include those suitable for oral, nasal, topical, transdermal, buccal, sublingual, rectal, vaginal or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient which may be combined with a carrier material to produce a single dosage form will generally be that amount of the composition
30 which produces a therapeutic effect. Generally, out of 100%, this amount will range from about 1% to about 99% of active ingredient, preferably from about 5% to about 70%, most preferably from about 10% to about 30%.

Methods of preparing these formulations or compositions include the step of bringing into association a composition of the present invention with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association a composition
5 of the present invention with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

Formulations of the invention suitable for oral administration may be in the form of capsules, cachets, pills, tablets, gelcaps, crystalline substances, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders,
10 granules, gel, partial liquid, spray, nebulae, mist, atomized vapor, aerosol, tincture, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) or as mouth washes and the like, each containing a predetermined amount of a
15 composition of the present invention as an active ingredient. A composition of the present invention may also be administered as a bolus, electuary, or paste.

In solid dosage forms of the invention for oral administration (capsules, tablets, pills, dragees, powders, granules, and the like), the active ingredient is mixed with one or more pharmaceutically acceptable carriers, such as sodium
20 citrate or dicalcium phosphate, or any of the following: fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, or silicic acid; binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose or acacia; humectants, such as glycerol; disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain
25 silicates, and sodium carbonate; solution retarding agents, such as paraffin; absorption accelerators, such as quaternary ammonium compounds; wetting agents, such as, for example, cetyl alcohol and glycerol monostearate; absorbents, such as kaolin and bentonite clay; lubricants, such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and
30 mixtures thereof; and coloring agents.

In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such

excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder
5 (for example, gelatin or hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered composition moistened with an inert liquid diluent.

10 The tablets, and other solid dosage forms of the pharmaceutical compositions of the present invention, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the
15 active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile, other polymer matrices, liposomes or microspheres.

They may be sterilized by, for example, filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid
20 compositions which may be dissolved in sterile water, or some other sterile injectable medium immediately before use.

These compositions may also optionally contain opacifying agents and may be of a composition that they release the active ingredient(s) only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a
25 delayed manner. Examples of embedding compositions which may be used include polymeric substances and waxes. The active ingredient may also be in micro-encapsulated form, if appropriate, with one or more of the above-described excipients.

Liquid dosage forms for oral administration of the compounds of the
30 invention include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as

ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, the oral compositions may also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

Suspensions, in addition to the active compounds, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

Formulations of the pharmaceutical compositions of the invention for rectal or vaginal administration may be presented in liquid or aerosol form, or as a suppository, which may be prepared by mixing one or more compounds of the invention with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active compound. Liquid or aerosol forms include, but are not limited to, gels, pastes, ointments, salves, creams, solutions, suspensions, partial liquids, sprays, nebulae, mists, atomized vapors, and tinctures. Formulations of the present invention which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate.

Formulations of the pharmaceutical compositions of the invention for nasal administration can be in solid, liquid, or aerosol form (e.g., powder, crystalline substance, gel, paste, ointment, salve, cream, solution, suspension, partial liquid, spray, nebulae, irrigant, wash, mist, atomized vapor or tincture).

Dosage forms for the topical or transdermal administration of a composition of this invention include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The composition may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

The ointments, pastes, creams and gels may contain, in addition to an composition of this invention, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

5 Powders and sprays may contain, in addition to a composition of this invention, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays may additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

10 Transdermal patches have the added advantage of providing controlled delivery of a composition of the present invention to the body. Such dosage forms may be made by dissolving or dispersing the composition in the proper medium. Absorption enhancers may also be used to increase the flux of the composition across the skin. The rate of such flux may be controlled by either providing a rate
15 controlling membrane or dispersing the composition in a polymer matrix or gel.

Ophthalmic formulations, eye ointments, powders, solutions and the like are also within the scope of this invention.

Pharmaceutical compositions of this invention suitable for parenteral administration comprise a composition of the invention in combination with one
20 or more pharmaceutically acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or
25 thickening agents.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable
30 organic esters, such as ethyl oleate. Proper fluidity may be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

In some cases, in order to prolong the effect of a cargo moiety, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally-administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable

to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, and sodium chloride, in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

5 Sterile injectable solutions can be prepared by incorporating a composition of the invention in the desired amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the composition into a sterile vehicle which contains a basic dispersion medium and the required
10 other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the compositions of the invention plus any additional desired ingredient from a previously sterile-filtered solution thereof.

15 Injectable depot forms are made by forming microencapsule matrices of the subject compounds in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release may be controlled. Examples of other biodegradable polymers include poly(orthoesters) and
20 poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissue.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the composition can be incorporated
25 with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the composition in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills,
30 capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium

stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compositions of the invention also can be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the compositions of the invention are prepared with carriers that will protect the composition against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity

of a composition calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the composition and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such a composition for the treatment of individuals.

The compositions can be included in a container, pack, or dispenser together with instructions for administration.

The compositions can be included in a container along with one or more additional compounds or compositions and instructions for use. For example, the invention also provides for packaged pharmaceutical products containing two agents, each of which exerts a therapeutic effect when administered to a subject in need thereof. A pharmaceutical composition may also comprise a third agent, or even more agents yet, wherein the third (and fourth, etc.) agent can be another agent against the disorder, such as a cancer treatment (*e.g.*, an anticancer drug and/or chemotherapy) or an HIV cocktail. In some cases, the individual agents may be packaged in separate containers for sale or delivery to the consumer. The agents of the invention may be supplied in a solution with an appropriate solvent or in a solvent-free form (*e.g.*, lyophilized). Additional components may include acids, bases, buffering agents, inorganic salts, solvents, antioxidants, preservatives, or metal chelators. The additional kit components are present as pure compositions, or as aqueous or organic solutions that incorporate one or more additional kit components. Any or all of the kit components optionally further comprise buffers.

The compositions of the instant invention may be used for delivering cargo moieties to food and drinks to be consumed by humans or other animals. For example, animal food (*e.g.*, human, cat, dog, fish, and bird food), can include the compositions of the present invention to stably deliver vitamins, minerals or other nutrients, as well as medications, *e.g.*, allergy medications and/or additional cargo moieties. The compositions of the present invention may be added to pet or domestic animal feed, such as fish food and food for fowl, cattle, and horses. The vehicles can be added at any step of the food preparation. For example, the formulations of the invention can be added at any point in the methods described

in WO 02/44026, incorporated herein by this reference. Similarly, the compositions and methods of the invention may be employed in food or drink to be consumed by humans, *e.g.*, in nutrient bars or drinks, cereals, breads, and snack foods. Accordingly, the preparations of the invention allow for the production of
5 stable, convenient preparations of micronutrients in processed foods, such as fast foods. Typically, potentially beneficial micronutrients, *e.g.*, omega fatty acids and antioxidants, can be destroyed during food manufacture and storage. The formulations of the invention may protect micronutrients and other cargo moieties, thus increasing the nutritional and/or medicinal value of the food.

10 The compositions and methods of the present invention can be added to foods that are baked or cooked, such as cakes, muffins, pasta noodles, soups, cereals, chips, candy and cookies. In some embodiments, the compositions are used in candy, such as candy bars, *e.g.*, chocolate bars. For example, omega fatty acid-compositions can be incorporated into a chocolate bar.

15 The present invention is useful in a variety of foods, including, dried food and beverage mixes, ready-to-drink and eat beverages and foods. These include baked good mixes and baked goods (*e.g.*, bread, cakes, brownies, muffins, cookies, pastries, pies, crackers, pie crusts), fried snacks derived from potatoes, corn, wheat and other grains (*e.g.*, potato chips, corn chips, tortilla chips), other
20 fried farinaceous snack foods (*e.g.*, french fries, doughnuts, fried chicken), dairy products and artificial dairy products (*e.g.*, butter, ice cream and other fat-containing frozen desserts, yogurt, and cheeses, including natural cheeses, processed cheeses, cream cheese, cottage cheese, cheese foods and cheese spread, milk, cream, sour cream, butter milk, and coffee creamer), cereal products, baby
25 foods or formulas, puddings, ice cream, dips, syrups, pie and other dessert fillings, frostings, emulsified spreads such as salad dressings, mayonnaise and margarines, various kinds of soups, dips, sauces and gravies. The preparations can include additional agents typically found in food preparations, such as coloring agents, flavoring agents, edible acids, preservatives, and the like.

30 The compositions of the present invention may be added to the food products in a crystallized or emulsion form at any stage of the manufacturing process. The compositions may be added at a stage and in a manner where the integrity of the delivery vehicle is maintained until ingestion, or final preparation

of the food product by the consumer. Another alternative, however, can be to use the compositions to maintain the stability of the agent until incorporation into the product, so activity can be maintained during storage and shipping. For example, food and drink mixes can contain compositions of the present invention that de-precipitate in whole or in part when reconstituted prior to ingestion. In this case, the compositions maintain the stability and integrity of the cargo moiety until ingestion so that the ingested food or drink contains the cargo moiety in a non-degraded state.

Yet another alternative is to deliver the formulations themselves to consumers or professionals, for direct addition to food products, *e.g.*, medicament, nutrient crystals, additives, supplements, or emulsions, such that one can vary the concentration as desired.

The compositions of the present invention may also be added to a carrier for use as a topical treatment on the skin. Suitable carriers would remain on the skin for an extended period of time, and be resistant to perspiration or immersion in water. Thus, for example, the formulations may be added to topical applications of medicaments, moisturizers, deodorants, balms, fragrances, sunscreens, and the like.

Additional examples of formulations that may include the compositions of the invention include, but are not limited to, hair care products, skin care products, personal care products, personal cleansing products, lotions, fragrances, sprays, perfumes, cosmetics, toothpastes, tooth whiteners, cleaners, bar soap, liquid soap, body wash, baby wash, makeup, hair color, shampoos, conditioners, styling products, balms, creams, solutions, gels and solids. Thus, for example, shampoos, conditioners and the like may contain the compositions of the invention loaded with vitamins, moisturizers, perfumes, medications, etc.

The compositions of the invention may also be added to cleansers which do not typically have prolonged direct contact with the skin. These formulations would be advantageous for, *i.e.*, the incorporation of perfumes, moisturizers or other such cargo moieties into fabric or for the introduction of an antibacterial agent to dishes. Examples include, but are not limited to, laundry detergent, pre-treating formulations, dryer sheets, fabric softener, and dishwashing detergent.

Compositions of the present invention may also be added to paper products for the topical application of cargo moieties to skin. Examples of paper products that can include compositions of the invention include baby care products, *i.e.*, diapers or baby wipes, tissues, toilet paper, antibacterial or antiperspirant
5 towelettes, napkins, paper towels, bandaids, gauze pads, and feminine hygiene products.

Exemplification

Example 1: Formation of Rigid Liposomes

10 Rigid liposomes were formed using distearoyl phosphatidylserine (DSPS) from Avanti Polar Lipids (Birmingham, AL), having a transition temperature of 68°C. Rhodamine-labeled Dextran (Rho-Dextran) in water was added to the DSPS in water at 0.1% by weight at room temperature.

15 The sample was heated to 70°C over a time period of 15 minutes while periodically vortexing to thoroughly mix the sample. Microscopic observations were made as the sample was warmed (Figures 1A and 1B). As the heating process began, the DSPS crystals slowly began to disappear and the tube became clear. After heating the sample above its transition temperature for approximately 15 minutes, the phospholipids were observed to form liposomes containing Rho-
20 Dextran (Figure 1B).

It was determined using fluorescence microscopy that the Rho-Dextran was within the DSPS liposomes, not free in the external aqueous space. Once the liposomes were formed, and the Rho-Dextran was found in the internal aqueous space of the DSPS liposomes, the sample was then cooled to 37°C, at which point
25 the liposomes became rigid. Calcium was then added causing an interaction that caused the composition to resemble a bunch or cluster of grapes (Figure 1C). It appeared as though the calcium formed a cationic bridge between the rigid liposomes, pulling them in together and forming a cluster of liposomes.

In the formation of cochleates from diolyl phosphatidylserine (DOPS),
30 which does not have a high transition temperature, the addition of EDTA chelates the calcium, such that the cochleate structure is unrolled and/or unstacked. In contrast, when EDTA was added to the DSPS sample at 37°C, no changes were

observed (Figure 1D). The sample remained rigid, and did not allow the EDTA to interact with the calcium.

When heated above the transition temperature of 68°C, however, the cationic matrix was observed to open up and the DSPS liposomes were observed
5 become fluid.

Example 2: Formation of Encochleated Rigid Liposomes

Rigid DSPS liposomes were formed in accordance with Example 1, except that the matrix was formed by forming a precipitate of DOPS and calcium. The DSPS lipid (10mg/ml) containing Rhodamine-labeled dextran, was added to water
10 and heated above 68°C while periodically vortexing. The heating of the lipid above its transition temperature allowed for the phospholipids to become fluid and small liposomes formed. The DSPS liposomes were then cooled to room temperature, at which point they became rigid as in Example 1. DSPS liposomes were added to pre-formed DOPS liposomes in water at a ratio of 1 to 10 by
15 weight, respectively. The sample was vortexed to ensure the rigid DSPS liposomes were evenly distributed, at which point the sample was observed microscopically. Microscopy of the sample revealed a population of two types of liposomes, those that were fluid and those that were rigid. The rigid DSPS liposomes were somewhat smaller than those composed of DOPS. After
20 microscopic observations indicated a heterogeneous population, calcium (0.1M) was added by dropwise addition while vortexing to allow formation of the cochleates. Upon addition of calcium, the DOPS liposomes, which were surrounding the DSPS rigid liposomes, interacted with the calcium. The calcium interaction caused a restructuring of the DOPS liposomes forcing out the water of
25 hydration, but did not appear to affect the structure of the rigid DSPS liposomes. Microscopic observation indicated that DOPS cochleates were formed, and appeared to be structurally similar to those that were prepared with the standard DOPS. When the sample was observed by fluorescence, it appeared that the rigid DSPS clusters liposomes were disposed in a DOPS cochleate matrix.

Figure 2A is an image of the formulation observed under phase contrast optical microscopy. Using both fluorescence and phase contrast microscopy, the rigid DSPS Rho-Dextran liposomes can be observed within the DOPS cochleate

structure as shown in Figure 2B. Turning down the phase contrast light, numerous fluorescent DSPS Rho-Dextran liposomes were observed within the cochleate structure as captured in Figure 2C.

Equivalents

- 5 Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

We claim:

1. A composition comprising a plurality of liposomes having a high transition temperature at least partially disposed in a matrix.
- 5 2. The composition of claim 1, wherein the transition temperature of the plurality of liposomes is at least about 30°C.
3. The composition of claim 1, wherein the transition temperature of the plurality of liposomes is at least about 45°C.
- 10 4. The composition of claim 1, wherein the transition temperature of the plurality of liposomes is at least about 60°C.
- 15 5. The composition of claim 1, wherein plurality of liposomes comprises at least one phospholipid selected from the group consisting of: distearoyl phosphatidylserine (DSPS), distearoyl phosphatidylcholine (DSPC), dipalmitoyl phosphatidic acid (DPPA), dipalmitoyl phosphatidylcholine (DPPC), dipalmitoyl phosphatidylethanolamine (DPPE), dipalmitoyl phosphatidylserine (DPPS), dipalmitoyl phosphatidylglycerol (DPPG), dimyristoyl phosphatidylethanolamine (DMPE), dioleoyl phosphatidic acid (DOPA), and dimyristoyl phosphatidylserine (DMPS).
- 20 6. The composition of claim 1, wherein the plurality of liposomes comprises a negatively charged lipid, and the matrix is a cationic bridge between a multivalent cation and at least a portion of the negatively charged lipid.
7. The composition of claim 1, wherein the matrix is a precipitate comprising a multivalent cation and a negatively charged lipid.
- 30 8. The composition of claim 1, wherein the matrix comprises a cochleate comprising a multivalent cation and a negatively charged lipid.

9. The composition of any of claims 1-8, wherein the composition comprises a cargo moiety.
10. The composition of claim 9, wherein the cargo moiety is associated with at least a portion of the plurality liposomes.
11. The composition of claim 10, wherein the cargo moiety is disposed within at least a portion of the plurality liposomes.
12. The composition of claim 9, wherein the cargo moiety is associated with the matrix.
13. The composition of claim 9, wherein the composition comprises at least one additional cargo moiety.
14. The composition of claim 9, wherein the cargo moiety is at least one member selected from the group consisting of a vitamin, a mineral, a nutrient, a micronutrient, an amino acid, a toxin, a microbicide, a microbistat, a co-factor, an enzyme, a polypeptide, a polypeptide aggregate, a polynucleotide, a lipid, a carbohydrate, a nucleotide, a starch, a pigment, a fatty acid, a saturated fatty acid, a monounsaturated fatty acid, a polyunsaturated fatty acid, a flavoring, an essential oil or extract, a hormone, a cytokine, a virus, an organelle, a steroid or other multi-ring structure, a saccharide, a metal, a metabolic poison, an antigen, an imaging agent, a porphyrin, a tetrapyrrolic pigment, and a drug.
15. The composition of claim 14, wherein the drug is at least one member selected from the group consisting of a protein, a small peptide, a bioactive polynucleotide, an antibiotic, an antiviral, an anesthetic, an antipsychotic, an anti-infectious, an antifungal, an anticancer, an immunosuppressant, an immunostimulant, a steroidal anti-inflammatory, a non-steroidal anti-inflammatory, an antioxidant, an antidepressant which can be synthetically or naturally derived, a substance which supports or enhances mental

- function or inhibits mental deterioration, an anticonvulsant, an HIV protease inhibitor, a non-nucleophilic reverse transcriptase inhibitor, a cytokine, a tranquilizer, a mucolytic agent, a dilator, a vasoconstrictor, a decongestant, a leukotriene inhibitor, an anti-cholinergic, an anti-histamine, a cholesterol lipid metabolism modulating agent, and a vasodilatory agent.
16. The composition of claim 14, wherein the drug is at least one member selected from the group consisting of Amphotericin B, acyclovir, adriamycin, carbamazepine, ivermectin, melphalen, nifedipine, indomethacin, curcumin, aspirin, ibuprofen, naproxen, acetaminophen, rofecoxib, diclofenac, ketoprofen, meloxicam, nabumetone, estrogens, testosterone, steroids, phenytoin, ergotamines, cannabinoids, rapamycin, propanadid, propofol, alphadione, echinomycin, miconazole, miconazole nitrate, ketoconazole, itraconazole, fluconazole, griseofulvin, clotrimazole, econazole, terconazole, butoconazole, oxiconazole, sulconazole, saperconazole, voriconazole, ciclopirox olamine, haloprogin, tolnaftate, naftifine, terbinafine hydrochloride, morpholines, flucytosine, natamycin, butenafine, undecylenic acid, Whitefield's ointment, propionic acid, and caprylic acid, clioquinol, selenium sulfide, teniposide, hexamethylmelamine, taxol, taxotere, 18-hydroxydeoxycorticosterone, prednisolone, dexamethazone, cortisone, hydrocortisone, piroxicam, diazepam, verapamil, vancomycin, tobramycin, teicoplanin, bleomycin, peptidoglycan, ristocetin, sialoglycoproteins, oritricin, avaporcine, hevecardin, galacardin, actinoidin, gentamycin, neilmicin, amikacin, kanamycin A, kanamycin B, neomycin, paromomycin, neamine, streptomycin, dihydrostreptomycin, apramycin, ribostamycin, spectinomycin, caspofungin, echinocandin B, aculeacin A, micafungin, anidulafungin, cilofungin, pneumocandin, geldanamycin, nystatin, rifampin, tyrphostin, a glucan synthesis inhibitor, vitamin A acid, mesalamine, risedronate, nitrofurantoin, dantrolene, etidronate, nicotine, amitriptyline, clomipramine, citalopram, dothepin, doxepin, fluoxetine, imipramine, lofepramine, mirtazapine, nortriptyline, paroxetine, reboxetine,

- sertraline, trazodone, venlafaxine, dopamine, St. John's wort, phosphatidylserine, phosphatidic acid, amastatin, antipain, bestatin, benzamidine, chymostatin, 3,4-dichloroisocoumarin, elastatinal, leupeptin, pepstatin, 1,10-phenanthroline, phosphoramidon, ethosuximide, ethotoin, felbamate, fosphenytoin, lamotrigine, levitiracetam, mephenytoin, methsuximide, oxcarbazepine, phenobarbital, phensuximide, primidone, topiramate, trimethadione, zonisamide, saquinavir, ritonavir, indinavir, nelfinavir, and amprenavir.
- 10 17. The composition of claim 14, wherein the polynucleotide is at least one member selected from the group consisting of a deoxyribonucleic acid (DNA) molecule, a ribonucleic acid (RNA) molecule, small interfering RNA (siRNA), a ribozyme, an antisense molecule, a morpholino and a plasmid.
- 15 18. The composition of claim 14, wherein the DNA is transcribed to yield a ribonucleic acid.
- 20 19. The composition of claim 18, wherein the ribonucleic acid is translated to yield a biologically active polypeptide.
- 25 20. The composition of claim 14, wherein the polypeptide is at least one member selected from the group consisting of cyclosporin, Angiotensin I, II, or III, enkephalins and their analogs, ACTH, anti-inflammatory peptides I, II, or III, bradykinin, calcitonin, beta-endorphin, dinorphin, leucokinin, leutinizing hormone releasing hormone (LHRH), insulin, neurokinins, somatostatin, substance P, thyroid releasing hormone (TRH), and vasopressin.
- 30 21. The composition of claim 14, wherein the antigen is at least one member selected from the group consisting of a membrane protein, a carbohydrate, envelope glycoproteins from viruses, an animal cell protein, a plant cell protein, a bacterial protein and a parasitic protein.

22. The method of claim 14, wherein the nutrient is at least one member selected from the group consisting of vitamins, minerals, fatty acids, amino acids, fish oils, fish oil extracts, resveratrol, biotin, choline, inositol, ginkgo, 5 saccharides, a phytochemical or zoochemical, beta-carotene, lutein, zeaxanthine, quercetin, silibinin, perillyl alcohol, genistein, sulfurophane, lycopene, essential fatty acids, eicosapentanoic acid (EPA), gamma-3, omega-3, gamma-6, and omega-6 fatty acids.
- 10 23. The composition of claim 14, wherein the vitamin is at least one member selected from the group consisting of vitamins A, B, B1, B2, B3, B12, B6, B-complex, C, D, E, and K, vitamin precursors, carotenoids, and beta-carotene.
- 15 24. The composition of claim 14, wherein the mineral is at least one member selected from the group consisting of boron, chromium, colloidal minerals, colloidal silver, copper, manganese, potassium, selenium, vanadium, vanadyl sulfate, calcium, magnesium, barium, iron and zinc.
- 20 25. The composition of claim 14, wherein the fatty acid is at least one member selected from the group consisting of monounsaturated, polyunsaturated and saturated fatty acids.
26. The composition of claim 14, wherein the saccharide or sweetener is at 25 least one member selected from the group consisting of saccharine, isomalt, maltodextrine, aspartame, glucose, maltose, dextrose, fructose and sucrose.
27. The composition of claim 14, wherein the flavor substance is an essential 30 oil or an extract.
28. The composition of claim 14, wherein the flavor substance is selected from the group consisting of oils and extracts of cinnamon, vanilla,

almond, peppermint, spearmint, chamomile, geranium, ginger, grapefruit, hyssop, jasmine, lavender, lemon, lemongrass, marjoram, lime, nutmeg, orange, rosemary, sage, rose, thyme, anise, basil, black pepper and tea or tea extracts.

5

29. The composition of claim 28, wherein the extract is from at least one member selected from the group consisting of an herb, a citrus, a spice and a seed.

10 30. A pharmaceutical composition comprising the composition of any of claims 1-29 and a pharmaceutically acceptable carrier.

31. A method of treating a subject that can benefit from administration of a cargo moiety comprising administering the composition of any one of
15 claims 9-29 such that the cargo moiety is administered to the subject.

32. The method of treatment according to claim 31, wherein the administration is by a mucosal or a systemic route.

20 33. The method of treatment according to claim 31, wherein the administration is a mucosal route selected from the group consisting of oral, intranasal, intraocular, intrarectal, intravaginal, and intrapulmonary.

34. The method of treatment according to claim 31, wherein the administration
25 is by a systemic route selected from the group consisting of intravenous, intramuscular, subcutaneous, transdermal, and intradermal.

35. The method of claim 31, wherein the cargo moiety is administered to treat inflammation, pain, infection, fungal infection, bacterial infection, viral
30 infection, parasitic disorders, an immune disorder, genetic disorders, degenerative disorders, cancer, proliferative disorders, obesity, depression, hair loss, impotence, hypertension, hypotension, dementia, senile dementia, or malnutrition, acute and chronic leukemia and lymphoma,

- 5 sarcoma, adenoma, carcinomas, epithelial cancers, small cell lung cancer, non-small cell lung cancer, prostate cancer, breast cancer, pancreatic cancer, hepatocellular carcinoma, renal cell carcinoma, biliary cancer, colorectal cancer, ovarian cancer, uterine cancer, melanoma, cervical cancer, testicular cancer, esophageal cancer, gastric cancer, mesothelioma, glioma, glioblastoma, pituitary adenomas, schizophrenia, obsessive compulsive disorder (OCD), bipolar disorder, Alzheimer's disease, Parkinson's disease, cell proliferative disorders, blood coagulation disorders, Dysfibrinogenaemia and hemophilia (A and B), autoimmune disorders, *e.g.*, systemic lupus erythematosus, multiple sclerosis, myasthenia gravis, autoimmune hemolytic anemia, autoimmune thrombocytopenia, Grave's disease, allogenic transplant rejection, ankylosing spondylitis, psoriasis, scleroderma, uveitis, eczema, dermatological disorders, hyperlipidemia, hyperglycemia, or hypercholesterolemia.
- 10
- 15
36. A method of forming a composition comprising the step of forming a matrix at least partially about a plurality of liposomes having a high transition temperature.
- 20
37. The method of claim 36, wherein the plurality of liposomes comprise an anionic lipid and the matrix comprises a multivalent cation.
38. The method of claim 36, wherein the matrix comprises a multivalent cation forming a cationic bridge between a portion of the plurality of liposomes.
- 25
39. The method of claim 36, wherein the matrix comprises a precipitate of a multivalent cation and a second plurality of liposomes.
- 30
40. The method of claim 36, wherein the matrix comprises a cochleate comprising a multivalent cation and a second plurality of liposomes.

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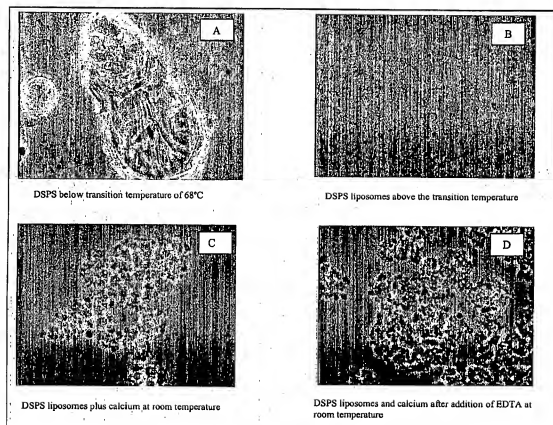
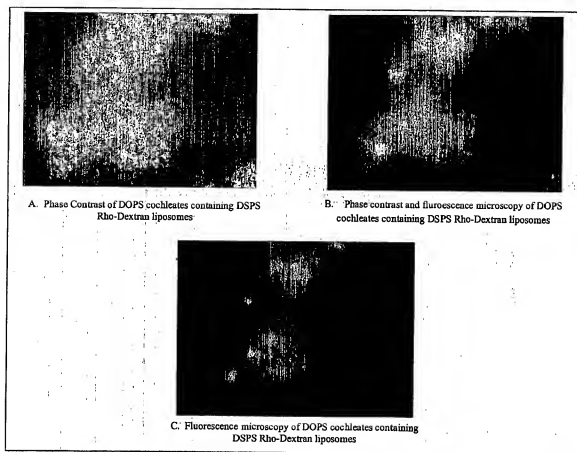
Figure 1

Figure 2



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2004/042927

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K9/127 A61K47/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EP0-Internal, WPI Data, PAJ, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

 International Application No
 PCT/US2004/042927

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US2004/042927

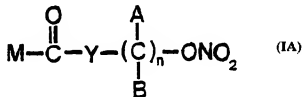
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(71) Applicant (for all designated States except US): NICOX LIMITED [IE/IE]; 17 Dame Street, Dublin 2 (IE).			
(72) Inventor; and (75) Inventor/Applicant (for US only): DEL SOLDATO, Piero [IT/IT]; Via E. Toti, 22, I-20052 Monza (IT).			
(74) Agent: TRUPIANO, Roberto; Brevetti Europa S.r.l., Piazza Bernini, 6, I-20133 Milano MI (IT).		Published <i>With international search report.</i>	

(54) Title: **NITRIC ESTERS HAVING ANTI-INFLAMMATORY AND/OR ANALGESIC ACTIVITY AND PROCESS FOR THEIR PREPARATION**



(57) Abstract

The present invention refers to nitric esters of derivatives of propionic acid, 1-(p-chlorobenzoyl)-5-methoxy-2-methyl-3-indolylacetic acid, 5-benzoyl-1,2-dihydro-3H-pyrrrolo[1,2-a]pyrrole-1-carboxylic acid, 6-methoxy-2-naphthylacetic acid, having general formula (IA), their pharmaceutical use and the process for their preparation.

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NITRIC ESTERS HAVING ANTI-INFLAMMATORY AND/OR ANALGESIC ACTIVITY AND PROCESS FOR THEIR PREPARATION.

OBJECT OF THE INVENTION

The present invention refers to nitric esters of derivatives of propionic acid, 1-(p-chlorobenzoyl)-5-methoxy-2-methyl -3-indolylacetic acid, 5-benzoyl -1,2-dihidro -3H- pyrrolo[1,2-a]pyrrole -1-carboxylic acid, 6-methoxy -2-naphthylacetic acid, their pharmaceutical utilization and the process for their preparation. The present invention also refers to pharmaceutical compositions comprising at least one of said nitric esters as active constituent.

PRIOR ART

Some derivatives of propionic acid, such as, for instance, 2-(6-methoxy-2-naphtyl)propionic acid 2-(4-isobutylphenyl)propionic acid or alpha-Methyl-4-[(2-oxocyclopentyl)methyl]benzeneacetic acid, have been used for a long time in the pharmaceutical field for their anti-inflammatory activity and have been present for many years on the different world markets. The process for the preparation of 2-(6-methoxy-2-naphtyl)propionic acid has been described in the South African Patent N°6707,597, in the German Patent N° 1,934,460, corresponding to the US Patent N°3,637,767 and also in C.A.71,91162j (1969); HARRISON et al. J.Med.Chem. 13,203 (1970); the process for the preparation of 2-(4-isobutylphenyl)propionic acid has been

described in Patents GB N°971,700, US N°3,228,831 and US N°3,385,886, and also in T. SHIORI, N. KAWAI, J.Org. Chem. 43,2936 (1978); J.T. PINHEY, B.A. ROWE, Tetrahedron Letters 21, 965 (1980); while the process for the preparation of alpha-methyl-4-[(2-oxocyclopentyl)methyl]benzenacetic acid has been described in the German Patent N°2,814,556 and in US Patent N°4,161,538.

In the case of 2-(6-methoxy-2-naphtyl)propionic acid, the pharmacological profile is described in ROSZKOWSKI et al. J. Pharmacol. Exp. Ther. 179,114 (1971), while the pharmacological profile of 2-(4-isobutylphenyl)propionic acid is reported in ADAMS et al. Arch. Pharmacodyn. Ther. 178,115 (1969).

The utilization of these derivatives of propionic acid as anti-inflammatory agents involves, as known, extremely severe adverse reactions affecting, for instance, the gastrointestinal system, as well as damages to liver and kidneys.

Other particularly toxic products are, for example, 5-benzoyl -,2- dihydro-3H- pyrrolo[1,2-a] pyrrole 1-carboxylic acid or Ketorolac [W.H.ROOKS et al. Agents Actions 12,684 (1982)] and 1-(4-chlorobenzoyl)-5-methoxy-2- methyl- 1H-indole- 3-acetic acid or Indomethacin [C.D.KLAASSEN, Toxicol. Appl.Pharmacol. 28,127 (1976)]. In particular, in some countries Ketorolac has been withdrawn from the market because of its gastrointestinal toxicity, while Indomethacin is one of the

drugs which has caused the highest death-rate from the year of its introduction in the market. Compared with other known anti-inflammatory and/or analgesic drugs, Ketorolac and Indomethacin cause - because of the already described adverse reactions - very extensive damages and, in particular as concerns gastrointestinal toxicity, deaths have been ascertained even in children.

It is therefore evident that there is the need of having drugs which, though providing a good anti-inflammatory and/or analgesic activity, do not result to be, in general, toxic.

OBJECTS OF THE INVENTION

Object of the present invention is that of providing a product which, while assuring at least the maintenance of the pharmacological activity which is characteristic of the known anti-inflammatory and/or analgesic agents, is capable of eliminating the adverse reactions brought about by the treatment with said agents, and has good tolerance.

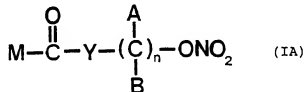
Another object of the present invention is that of realizing a process for the preparation of derivatives of propionic acid, 1-(p-chlorobenzoyl)-5-methoxy-2-methyl -3-indolylacetic acid, 5-benzoyl -1,2-dihydro -3H- pyrrolo[1,2-a]pyrrole -1-carboxylic acid, 6-methoxy -2-naphthylacetic acid, having an anti-inflammatory and/or analgesic activity, good tolerance and being

exempt from the adverse reactions that are typical of anti-inflammatory and analgesic agents.

Still another object of the present invention is that of providing pharmaceutical compositions having anti-inflammatory and/or analgesic activity which results provided with good tolerance.

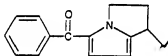
DESCRIPTION OF THE INVENTION

These and still further objects and associated advantages which shall clearly result from the following description, are reached by derivatives of propionic acid, 1-(p-chlorobenzoyl)-5-methoxy-2-methyl-3-indolylacetic acid, 5-benzoyl-1,2-dihydro-3H-pyrrolo[1,2-a]pyrrole-1-carboxylic acid, 6-methoxy-2-naphthylacetic acid which, according to the present invention, have the following general formula:

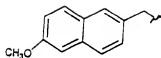


where:

A and B are chosen among hydrogen, linear or branched, substituted or non substituted alkyl chains, M is chosen among:

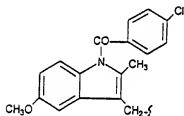


(XXX)



(XXXI)

5



(XXXII)

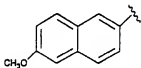
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(XXXIII)

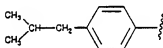
where R is chosen among:

15

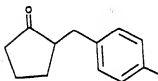


(II)

20



(III)



(X)

25

Y is chosen among oxygen, NH, NR₁, where R₁ is a linear

or branched alkyl group, and n is comprised between 1 and 10.

More particularly, the fragment

5



is a linear, branched or cyclic alkylene group $\text{C}_2\text{-C}_{10}$. In fact, it has been observed that the introduction of a group such as a terminal nitric ester in the derivatives (IA) permits to maintain the pharmacological activity which is characteristic of anti-inflammatory non steroidal and/or analgesic agents, leads to products provided with good tolerance, while eliminating the adverse reactions caused by the treatment with such drugs. Furthermore, the introduction of a terminal nitric ester in the derivatives of propionic acid, permits to potentiate the anti-inflammatory effect compared with the well known non-steroidal anti-inflammatory drugs; such increase is made by the terminal nitric ester group, which can be considered as a source of nitric oxide and which can exert additional anti-inflammatory effects.

It has been also observed that the derivatives (IA) are useful in the treatment of different unhealthy conditions, for instance unhealthy conditions which required the treatment with both anti-inflammatory and analgesic drug, or rheumatic diseases in general, disorders of an

immunologic nature, and they can also alleviate moderate-medium painful states of any kind.

Moreover, the derivatives (IA) subject matter of this invention, are useful in the treatment of the illnesses of the cardiovascular system and of the central nervous system, in particular in the treatment of myocardial and brain ischaemiae, as well as in some cases of arterial thrombosis and in some cases of senile dementia.

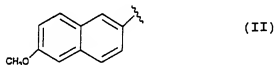
Always according to this invention, a nitric ester (IA)

proved to be particularly advantageous, where:

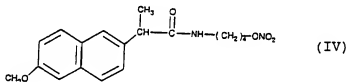
hydrogen is chosen as A and B, M is chosen as



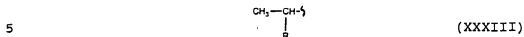
where R is chosen as:



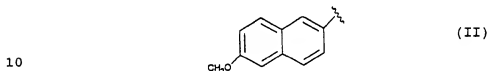
NH is chosen as Y, and n is equal to four, according to the following formula:



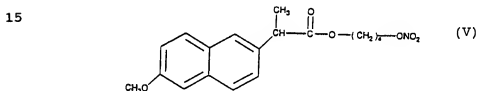
A nitric ester (IA) has also proved to be particularly advantageous according to this invention, where:
hydrogen is chosen as A and B, M is chosen as



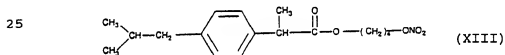
where R is chosen as:



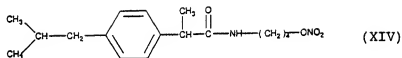
oxygen is chosen as Y, an n is equal to four, according to the following formula:



20 Also the nitric esters of derivatives of 2-(4-isobutylphenyl)propionic acid have proved to be particularly advantageous according to this invention, having the following formulae:

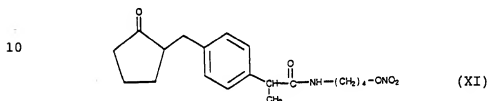


and

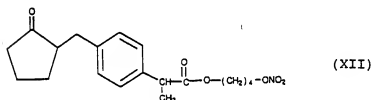


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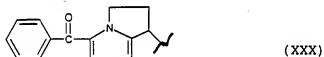
Always according to the present invention, nitric esters (IA) have proved to be particularly advantageous, having the following formulae:



15



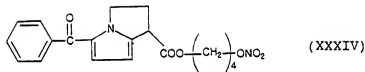
20 Always according to the present invention, nitric esters (IA) where M is chosen as



25

oxygen is chosen as Y, hydrogen is chosen as A and B and n is equal to four according to the following

formula:



5

proved to have very good tolerance.

For the preparation of nitric esters (IA) subject matter of the present invention, a first process has proved to be particularly advantageous which, according

to the present invention, includes the following steps:

- Preparation of sodium salt of derivatives having the following general formula:



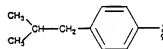
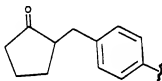
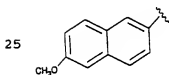
15

where M is chosen among (XXX), (XXXI), (XXXII),



20

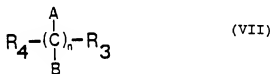
where R is chosen among the following structures:



25

or preparation of derivatives (VIA) functionalized to the carboxylic group as acyclic chlorides, anhydrides or the like;

- Reaction between the sodium salt of said derivatives (VIA) or of said derivatives (VIA) functionalized to the carboxylic group, with a composition having the following general formula:



where:

R_4 is chosen among chlorine, bromine, NHR_5 with R_5 hydrogen, linear or branched alkyl chain, A and B are chosen among hydrogen, linear or branched, substituted or non substituted alkyl chains, R_3 is chosen among chlorine, bromine and iodine, and n is comprised between 1 and 10, with ensuing production of the relevant monomeric esters or the relevant amides;

- Reaction of said monomeric esters or said amides with a nitrating agent such as AgNO_3 or the like, with ensuing production of nitric esters (IA).

A second process has also proved to be particularly advantageous which, always according to the present invention, includes the following steps:

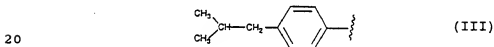
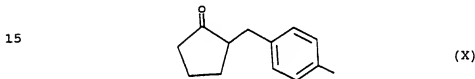
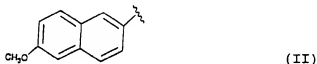
- Preparation of sodium salt of derivatives having the following general formula:



5 where M is chosen among (XXX), (XXXI), (XXXII),



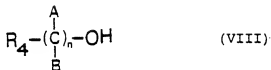
10 where R is chosen among the following structures:



or preparation of derivatives (VIA) functionalized to the carboxylic group, such as acyclic chlorides, anhydrides or the like;

25 - Reaction between the sodium salt of said derivatives (VIA) or of said derivatives (VIA) functionalized to the carboxylic group, with a composition having the

following general formula:



5

where;

R_4 is chosen among chlorine, bromine, NHR_5 with R_5 hydrogen, linear or branched alkyl chains, A and B are chosen among hydrogen, linear or branched, substituted
10 or non substituted alkyl chains, and n is comprised between 1 and 10, with ensuing production of the relevant monomeric esters or the relevant amides;

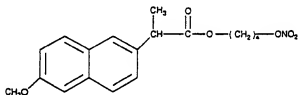
- Reaction of said monomeric esters or said amides with an halogenating composition such as PBr_3 or the like,
15 with ensuing prouction of said monomeric esters or said amides characterized by the presence of a terminal halogen group;

- Reaction of said monomeric esters or said amides characterized by the presence of a terminal halogen
20 group, with a nitrating agent such as AgNO_3 or the like, with ensuing production of nitric esters of derivatives (IA).

The solvents which are utilized in the processes subject matter of the present invention are preferably
25 chosen among chloroform, methylene chloride, acetonitrile, dimethylformamide, tetrahydrofuran, 1,4-dioxane and the like.

Such processes for the preparation of derivatives (IA), subject matter of the present invention, consist of a limited number of steps, which permits to obtain in a short time the products which derive from these processes, with satisfactory yields and in high amounts, also on the industrial level.

According to the processes subject matter of this invention, the preparation of a nitric ester derived from propionic acid has proved to be particularly advantageous, having the following formula:



(V)

which is prepared as described in the example that is given hereunder as a mere indication and which does not limit in any way the protection scope of the invention.

EXAMPLE 1

a) 0.59 g of EtONa dissolved in 10 ml of ethyl alcohol were added, by slow dripping, to a solution of 2 g of 2-(6-methoxy-2-naphtyl)propionic acid, dissolved in 20 ml of ethyl alcohol. The reaction mixture was stirred for 5 minutes at room temperature, then the solvent was evaporated at a reduced pressure, obtaining 2.1 g of sodium salt of 2-(6-methoxy-2-naphtyl)propionic acid. The 2.1 g of sodium salt of 2-(6-methoxy-2-naphtyl)

propionic acid so obtained were dispersed in 40 ml of dimethylformamide and 1.5 g of 1-Br-4-Cl-butane dissolved in 30 ml of dimethylformamide were added by dripping to this dispersion. The reaction mixture was stirred for 12 hours at room temperature, then diluted with water and extracted with methylene chloride. The organic phase so extracted was anhydriified on sodium sulfate and the solvent was evaporated at a reduced pressure until a dry residue of 2 g was obtained.

The residue was purified by chromatography on silica gel, utilizing an eluting mixture constituted by hexane/ether 7/3 (v/v).

The head fractions were collected, the solvent was evaporated at a reduced pressure and 1 g of 2-(6-methoxy-2-naphtyl)propionate of 4-chlorobutyl (IX) was obtained.

IR(cm^{-1}): C=O, 1669.

$^1\text{H-NMR}$ (300MHz) (CDCl_3): 1.6ppm (d, 3H); 1.75ppm (m, 4H); 3.45ppm (m, 2H); 3.88ppm (q, 1H); 3.91ppm (1, 3H); 4.1ppm (m, 2H); 7.1-7-7.7ppm (m, aromatics).

Mass spectrometry (i.e.): M^+ 320.

b) 0.79 g of AgNO_3 dissolved in 1.3 ml of acetonitrile were dripped to 1 g of (IX) obtained as described in a), dissolved in 4,5 ml of acetonitrile. The reaction mixture was stirred for 12 hours at a temperature of 85°C and then filtered.

From the resulting solution, the solvent was evaporated

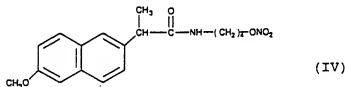
at a reduced pressure, and a residue was obtained to which 10 ml of methylene chloride were added. The mix so obtained was filtered once again, the organic phase was washed with water and then anhydried on sodium sulfate. The solvent was evaporated under reduced pressure and 1.8 g of a dry residue was obtained, which was purified by chromatography on silica gel, utilizing an eluting mixture constituted by hexane/ether 7/3 (v/v). The fractions containing the product were collected, the solvent was evaporated at a reduced pressure and 1.5 g of nitric ester of 2-(6-methoxy-2-naphthyl)propionate of 4-hydroxy-butyl (V) were obtained.

IR(cm^{-1}): C=O, 1733; ONO_2 , 1637.

$^1\text{H-NMR}$ (300MHz) (CDCl_3): 1.6ppm (d, 3H); 1.65ppm (m, 4H); 3.8ppm (q, 1H); 3.9ppm (s, 3H); 4.1ppm (m, 2H); 4.3ppm (m, 2H); 7.1-7.7ppm (m, aromatics).

Mass spectrometry (i.e.) M^+ 347.

Always according to the processes subject matter of the present invention, also the preparation of a nitric ester derivated from propionic acid proved to be particularly advantageous, having the following formula:



which is prepared as described in the following example, that is given hereunder as a mere indication and

which does not limit in any way the protection scope of this invention.

EXAMPLE 2

5 a) 23.9 g of potassium-phthalimide dispersed into 200 ml of anhydrous dimethylformamide were added to a solution of 55.7 g of 1,4-di bromo-butane dissolved in 300 ml of anhydrous dimethylformamide.

The reaction mixture was agitated for 12 hours at room temperature, then diluted with water and extracted with
10 methylene chloride. The methylene chloride was evaporated from the organic phase so obtained at a reduced pressure and then the dimethylformamide was removed by distillation at the pressure of 10 mm Hg.

The residue was regained with water and extracted with
15 methylene chloride.

The organic phase so obtained was anhydriified and the solvent was evaporated at a reduced pressure until 14.8 g of 1-phthalimide-4-bromo-butane were obtained, which were treated with isopropyl ether and then essiccated.
20 m.p. = 77°C

b) 32 ml of hydriodic acid were cautiously added to 8.25 g of 1-phthalimido-4-bromo-butane; the mixture was then submitted to heating and kept in ebullition for 24 hours.

25 After cooling, the mixture was diluted with water and after filtration the solvent was evaporated at a reduced pressure, obtaining a residue which, once crystal-

lized by ethyl ether, produced 6 g of 4-iodine-butylammonium iodide.

m.p. = 103°C

5 c) 7 ml of thionyl chloride were cautiously added to a solution of 2.3 g of 2-(6-methoxy-2-naphtyl)propionic acid in 15 ml of anhydrous chloroform. The reaction mixture was stirred for 40 minutes at room temperature and then the solvent was evaporated at a reduced pressure, obtaining 2.23 g of 2-(6-methoxy-2-naphtyl)propionylchloride.

10 2.3 g of 2-(6-methoxy-2-naphtyl)propionylchloride were dissolved in pyridine and the solution was cooled at the temperature of 0°C.

3.27 g of 4-iodobutylammonium iodide were added to this solution and the mixture so obtained was agitated for 1 hour at 0°C and then diluted with water and extracted with methylene chloride.

The organic phase so obtained was washed initially with a 10% solution of hydrochloric acid and afterward with a saturated solution of sodium bicarbonate, then the solvent was evaporated at a reduced pressure, obtaining 3.2 g of a dry residue. The residue was purified by chromatography on silica gel, utilizing methylene chloride as eluent.

25 The intermediate fractions were collected, the solvent was evaporated at a reduced pressure and 1.6 g of 2-(6-methoxy-2-naphtyl)-4-iodobutyl propionamide (XX) were

obtained.

IR (cm⁻¹): NH, 3294; C=O, 1651.

¹H-NMR(300MHz) (CDCl₃): 1.1-1.75 ppm (m, 4H);

1.6ppm (d, 3H); 3.1ppm (t, 2H); 3.2ppm (q, 2H); 3.7ppm
5 (q, 1H); 3.9ppm (s, 3H); 5.35ppm (m, NH); 7.1-7.75ppm
(m, aromatics).

d) A suspension of 1.6 g of 2-(6-methoxy-2-naphtyl)-4-
iodobutyl propionamide in 20 ml of acetonitrile was
heated at a temperature of about 40°C and stirred until
10 a solution was obtained to which 1.0 g of AgNO₃ were
added.

The mixture was stirred for 1 hour at room temperature,
then filtered and the solvent was evaporated at a
reduced pressure. The residue obtained was regained
15 with methylene chloride, the resulting mixture was
filtered and the solvent was evaporated at a reduced
pressure, and 0,8 g of dry residue were obtained which
were purified by chromatography on silica gel, utili-
zing an eluting mixture constituted by methylene chlo-
20 ride/ethyl acetate 9/1 (v/v).

The head fractions were collected, the solvent was
evaporated at a reduced pressure and 0.75 g of nitric
ester of 2-(6-methoxy-2-naphtyl)-4-hydroxybutyl propio-
namide (IV) were obtained.

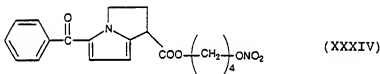
25 IR(cm⁻¹): C=O, 1672; NH, 3294; ONO₂, 1637

Mass spectrometry (i.e.) M⁺.346.

¹H-NMR(80mhz) (CDCl₃): 1.3ppm-1.6ppm (m, 4H);

1.7ppm (d, 3H); 3.1ppm (q, 2H); 3.7ppm (q, 1H); 3.9ppm (s, 3H); 4.3ppm (m, 2H); 5.6ppm (m, NH); 7.05-7.8ppm (m, aromatics).

Always according to the present invention, also the
5 nitric ester having the following formula:



10

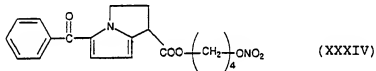
proved to be particularly advantageous, which is prepared as described in the following example that is also given hereunder as a mere indication and which does not limit in any way the protection scope of this invention.

15

EXAMPLE 3

Preparation of the composition having the formula:

20



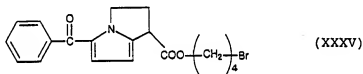
a) In a suspension of 80% sodium hydride (0,16 g) in DMF (15 ml), 1,15 g of Ketorolac dissolved in 20 ml of DMF were caused to drip under agitation.

25

The reaction mix was kept under agitation at 40°C for

15 minutes, then 1 ml of 1,4-dibromobutane was added and the mix was kept under agitation at room temperature overnight.

Then the solvent was evaporated under reduced pressure and the residue was treated with water and methylene chloride. The organic phase was separated, dried on sodium sulfate and the solvent was removed under reduced pressure, to obtain a residue which was purified by silica gel chromatography, utilizing a 4/6 petroleum ether/ether eluent mix (v/v). The head fractions were collected, the solvent was evaporated under reduced pressure and 0.75 g of product was obtained having the formula:



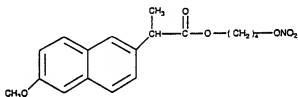
¹H-NMR (80 MHz) (CDCl₃) (ppm): 1,83(6H, m); 2,81(2H, m); 3,38(2H, t); 4,12(2H, t); 4,48(1H, m); 6,03(1H, d); 6,78(1H, d); 7,41(3H, m); 7,73(2H, m).

b) A solution of AgNO₃ (0,5 g) in 5 ml of acetonitrile was added to a solution of (XXXV) (0,75 g) in 20 ml of acetonitrile. The reaction mix was kept stirring at room temperature for 48 hours. The solvent was then removed under pressure and the residue was treated with water and methylene chloride. The organic phase

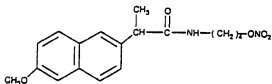
was then separated, dried on sodium sulfate and the solvent was removed under reduced pressure. The residue was purified by filtration on silica gel, utilizing a 4/6 petroleum ether/ether eluent mix. The head fractions were collected, the solvent was evaporated under reduced pressure and 0.35 g of (XXXIV) were obtained.

$^1\text{H-NMR}$ (80 MHz) (CDCl_3) (ppm): 1.78(6H, m); 2.82(2H, m); 4.14(2H, m); 4.47(3H, m); 6.03(1H, d); 6.79(1H, d); 7.46(3H, m); 7.77(2H, m).

Through biological assays the anti-inflammatory and analgesic activity were determined, for instance of nitric esters (IA) having the following formulae:



(V)



(IV)

The anti-inflammatory activity of said nitric esters of derivatives of propionic acid was determined in Wistar rats utilizing the method of carrageenan edema, as reported in C.A. WINTER, E. RISLEY, G.W. NUSS, Proc. Soc. Exp. Biol. Med. 111,544-547 (1962), while the

analgesic activity of said derivatives was determined in Swiss mice as reported by L.C. HENDERSHOT, J. FORSAITH, J.Pharmacol. Exp. Ter. 125,237-249 (1959).

5 The anti-inflammatory and analgesic activity of said derivatives resulted to be comparable to 2-(6-methoxy-2-naphtyl)propionic acid taken as a reference.

10 The anti-platelet aggregation activity of said derivatives was determined on human platelets. Platelets were incubated with the compounds for 10 min at 37°C prior to stimulation with trombin. The anti-platelet aggregation activity of said derivatives resulted to be comparable to 2-(6-methoxy-2-naphtyl)propionic acid taken as a reference.

15 Then, the acute toxicity of said derivatives (IV) and (V) was evaluated by oral administration of a single dose of each composition (IV) and (V), utilizing groups of 10 Swiss mice for each derivative.

20 The incidence of lethality and the onset of a toxic symptomatology were reported for an observation period of 14 days.

Even after the administration of a dose of 750 mg/kg of composition (IV) or composition (V) no apparent toxicity symptoms were observed in the treated animals.

25 Further biological assays were carried out in order to define the pharmaco-toxicological profile of the studied compounds, in particular of composition (V),

compared with 2-(6-methoxy-2-naphtyl)propionic acid taken as reference.

A. PHARMACODYNAMIC ACTIVITY

ACUTE MODELS

- 5 Rat carrageenan paw edema. On the basis of preliminary experiments, the compound (V) and 2-(6-methoxy-2-naphtyl)propionic acid prove to have a comparable efficacy; the effective dose is comprised in the range from 1 to 10 mg/kg p.o.

10 SUBACUTE MODELS

- Rat adjuvant arthritis. The animals treated for 19 running days (from the 3rd to the 20th day after the inducing injection) with composition (V) or with 2-(6-methoxy-2-naphtyl)propionic acid, both of them at doses of 3 mg/kg p.o., showed a significant and comparable reduction in arthritic symptomatology compared with controls.

B. GASTROINTESTINAL TOLERABILITY

- 20 Damage to the gastric mucosa of the rat. The compound (V) was studied in comparison with 2-(6-methoxy-2-naphtyl)propionic acid taken as reference, both of them at doses comprised between 3 and 30 mg/kg p.o.; the compound (V) proved to be significantly better tolerated than 2-(6-methoxy-2-naphtyl)propionic acid. 2-(6-methoxy-2-naphtyl)propionic acid already at 3 mg/kg caused gastric damages, and such effects resulted to be dose-dependent, while the compound (V) proved to be

well tolerated even at doses of 30 mg/kg.

C. GENERAL PHARMACOLOGY

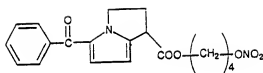
A secondary pharmacological evaluation of compound (V) was carried out in comparison with 2-(6-methoxy-2-naphtyl)propionic acid. No considerable additional effects with respect to the primary pharmacological activity were observed on central nervous system, on the autonomous system, on the cardiovascular, respiratory and gastrointestinal systems.

D. TOXICOLOGY

Acute toxicity in rodents. Preliminary studies were carried out in rodents, utilizing two administration routes. No symptoms of apparent toxicity were observed in animals treated with oral or intraperitoneal doses of 300 mg/kg.

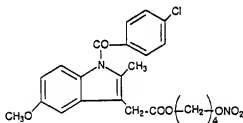
Maximum tolerated dose in non-rodents. Preliminary studies have indicated that compound (V) was very well tolerated in the dog, an animal species which is known to be particularly sensitive to the ulcerogenic activity of anti-inflammatory agents in general. The animals received increasing oral doses of compound (V) up to 30 mg/kg and no apparent symptoms were observed. In comparison, 2-(6-methoxy-2-naphtyl)propionic acid, administered at doses of 10 mg/kg, caused the death of the animals.

Furthermore, biological studies concerning nitric esters (IA) having the following formulae:



(XXXIV)

5



(XXXVI)

10

were carried out.

Then the anti-inflammatory activity, the gastrointestinal tolerability and the platelet anti-aggregating activity of the above compositions were determined.

15

The anti-inflammatory activity was determined by the method of the carrageenan edema in the rat, as described by C.A.WINTER et al. (1962) Proc.Soc.Exp.Biol.Med. 111,544. The gastrointestinal tolerability was evaluated by oral administration in the rat. The platelet anti-aggregating activity was determined on human platelets stimulated by arachidonic acid, according to the method described by V.BERTELE et al. (1983) Science 220, 517.

20

The results are shown on Table 1 as values concerning the anti-inflammatory, anti-aggregating activity and the gastrointestinal tolerability of the compositions

25

under examination, expressed as a power ratio relatively to the basic product taken as a unity standard.

TABLE 1

	COMPOSITION	ANTI-INFLAMM.	ANTI-AGGREG.	GASTROINTEST.
5		ACTIVITY	ACTIVITY	ULCEROGEN.
	(XXXIV)	1,25	1,10	0,15
	KETOROLAC	1,0	1,0	1,0
	(XXXVI)	1,0	1,30	0,1
	INDOMETHACIN	1,0	1,0	1,0

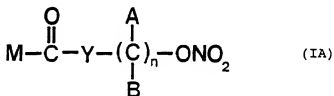
10 The acute toxicity of the compositions under examination has been approximately evaluated by oral administration of a single dosage of the substance to groups of 10 mice. The death-rate incidence and the onset of toxic symptoms have been observed for a period of 14
15 days. Even after the administration of 100 mg/kg of each composition, the animals did not show any symptom of apparent toxicity.

20

25

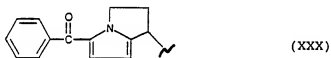
CLAIMS

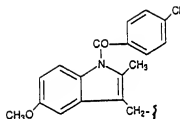
1. Derivatives of propionic acid, 1-(p-chlorobenzoyl)
 -5- methoxy-2-methyl -3-indolylacetic acid, 5-benzoyl
 -1,2-dihidro -3H- pyrrolo[1,2-a]pyrrole -1-carboxylic
 5 acid, 6-methoxy -2-naphthylacetic acid, characterized
 in that they have the following general formula:



where:

- A and B are chosen among hydrogen, linear or branched,
 substituted or non substituted alkyl chains, M is
 15 chosen among:



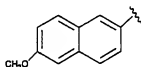


(XXXII)

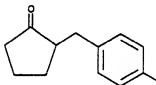


(XXXIII)

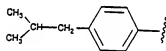
where R is chosen among:



(II)



(X)



(III)

Y is chosen among oxygen, NH, NR_1 , where R_1 is a linear or branched alkyl group, and n is comprised between 1 and 10.

2. Nitric esters according to claim 1, characterized in that the fragment:



5

is a linear, branched or cyclic alkylene group $\text{C}_2\text{-C}_{10}$.

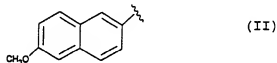
3. Derivative of propionic acid according to claim 1, characterized in that M is equal to

10



where R is:

15



A and B are equal to hydrogen, Y is equal to oxygen, and n is equal to four.

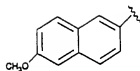
20

4. Derivative of propionic acid according to claim 1, characterized in that M is equal to

25



where R is:



(II)

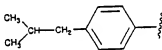
A and B are equal to hydrogen, Y is equal to NH, and n is equal to four.

5. Derivatives of propionic acid according to claim 1, characterized in that M is equal to



(XXXIII)

where R is equal to:



(III)

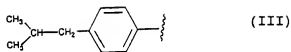
Y is equal to oxygen, A and B are equal to hydrogen, and n is equal to four.

6. Derivative of propionic acid according to claim 1, characterized in that M is equal to



(XXXIII)

where R is equal to:



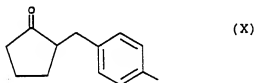
5

Y is equal to NH, A and B are equal to hydrogen, and n is equal to four.

7. Derivative of propionic acid, according to claim 1,
10 characterized in that M is equal to



15 where R is equal to



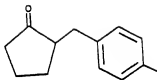
20 A and B are equal to hydrogen, y is equal to oxygen and n is equal to four.

8. Derivative of propionic acid according to claim 1,
characterized in that M is equal to

25



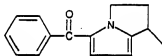
where R is equal to



(X)

A and B are equal to hydrogen, y is equal to NH and n is equal to four.

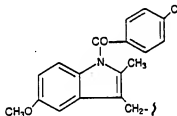
9. Derivatives of 5-benzoyl -1,2-dihydro-3H-pyrrolo[1,2-a] pyrrole -1-carboxylic acid according to claim 1, characterized in that M is equal to



(XXX)

A and B are equal to hydrogen, Y is equal to oxygen and n is equal to four.

10. Derivatives of 1-(p- chlorobenzoyl) -5-methoxy - 2-methyl-3-indolylacetic acid according to claim 1, characterized in that M is equal to



(XXXII)

A and B are equal to hydrogen, Y is equal to oxygen and

n is equal to four.

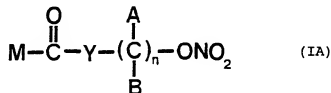
11. Nitric esters according to claim 1, characterized in that they are utilizable in the pharmaceutical field as anti-inflammatory agents.

5 12. Nitric esters according to claim 1, characterized in that they are utilizable in the pharmaceutical field as analgesic agents.

13. Nitric esters according to claim 1, characterized in that they are utilizable in the treatment of rheumatic illnesses, in the treatment of disorders of an immunologic nature and of the moderate to medium painful states.

14. Nitric esters according to claim 1, characterized in that they are utilizable in the treatment of the diseases of the cardiovascular system, in the treatment of senile dementia, in the treatment of miocardial and brain ischaemiae and in cases of arterial thrombosis.

15 15. Process for the preparation of nitric esters according to claim 1 and having the following general formula:
20

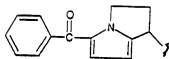


25

where A and B are chosen among hydrogen, linear or branched, substituted or non substituted alkyl chains,

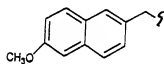
M is chosen among

5



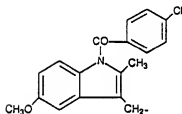
(XXX)

10



(XXXI)

15



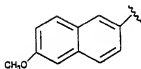
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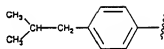
(XXXIII)

20 where R is chosen among:

25

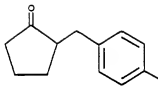


(II)



(III)

36



(X)

5

Y is chosen among oxygen, NH, NR_1 , where R_1 is a linear or branched alkyl group, and n is comprised between 1 and 10, characterized in that it comprises the following steps:

- 10 - Preparation of sodium salt of derivatives having the following general formula:



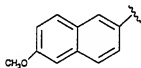
15

where M is chosen among (XXX), (XXXI), (XXXII),

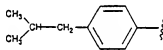


- 20 where R is chosen among the following structures:

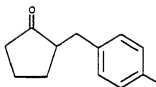
25



(II)



(III)



(X)

or preparation of derivatives (VIA) functionalized to the carboxylic group, such as acyclic chlorides, anhydrides or the like;

- Reaction between the sodium salt of said derivatives (VIA) or of said derivatives (VIA) functionalized to the carboxylic group, with a compound having the following general formula:

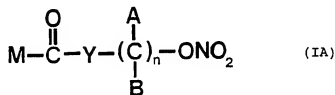


where:

R_4 is chosen among chlorine, bromine, NHR_5 with R_5 hydrogen, linear or branched alkyl chain, A and B are chosen among hydrogen, linear or branched, substituted or non substituted alkyl chains, R_3 is chosen among chlorine, bromine and iodine, and n is comprised between 1 and 10, with ensuing production of the relevant monomeric esters or the relevant amides;

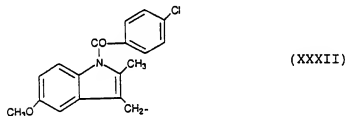
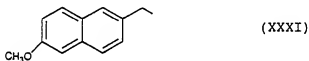
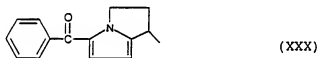
- Reaction of said monomeric esters or said amides with a nitrating agent such as AgNO_3 or the like, with ensuing production of nitric esters (IA).

16. Process for the preparation of nitric esters according to claim 1 and having the following general formula:



where:

A and B are chosen among hydrogen, linear or branched, substituted or non substituted alkyl chains, M is chosen among

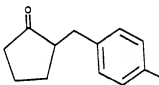




(XXXIII)

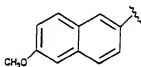
5

where R is chosen among:



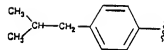
(X)

10



(II)

15



(III)

Y is chosen among oxygen, NH, NR_1 , where R_1 is a linear or branched alkyl group, and n is comprised between 1 and 10, characterized in that it comprises the following steps:

20

- Preparation of sodium salt of derivatives having the following general formula:



(VIA)

25

where M is chosen among (XXX), (XXXI), (XXXII),

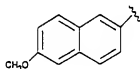
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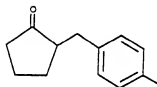
(XXXIII)

where R is chosen among the following structures:

10

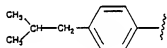


(II)



(X)

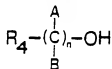
15



(III)

or preparation of derivatives (VIA) functionalized to the carboxylic group, such as acyclic chlorides, anhydrides or the like;

- Reaction between the sodium salt of said derivatives (VIA) or of said derivatives (VIA) functionalized to the carboxylic group, with a composition having the following general formula:



(VIII)

where:

R_4 is chosen among chlorine, bromine, NHR_5 with R_5 hydrogen, linear or branched alkyl chain, A and B are chosen among hydrogen, linear or branched, substituted or non substituted alkyl chains, and n is comprised between 1 and 10, with ensuing production of the relevant monomeric esters or the relevant amides;

- Reaction of said monomeric esters or said amides with an halogenating compound such as PBr_3 or the like, with ensuing production of said monomeric esters or said amides, characterized by the presence of a terminal halogen group;

- Reaction of said monomeric esters or of said amides, characterized by the presence of a terminal halogen group with a nitrating agent such as $AgNO_3$ or the like, with ensuing production of nitric esters (IA).

17. Pharmaceutical compositions having anti-inflammatory activity characterized in that they comprise at least one nitric ester according to claim 1 as active constituent.

18. Pharmaceutical compositions having analgesic activity characterized in that they comprise at least one nitric ester according to claim 1 as active constituent.

INTERNATIONAL SEARCH REPORT

 International Application No.
 PCT/EP 94/03182

A. CLASSIFICATION OF SUBJECT MATTER

 IPC 6 C07C203/04 C07D487/04 C07D209/28 A61K31/40 A61K31/405
 A61K31/21 //(C07D487/04, 209:00, 209:00)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07C C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DE,A,17 93 828 (SYNTEX CORP.) 22 April 1976 see the whole document & ZA,A,6 707 597 (...) cited in the application ---	1-18
A	DE,A,14 43 429 (BOOTS PURE DRUG COMPANY LTD.) 24 October 1968 see the whole document & GB,A,971 700 (...) cited in the application ---	1-18
A	US,A,3 758 544 (SYNTEX CORP.) 11 September 1973 see abstract; claims & DE,A,19 34 460 (...) 23 June 1977 cited in the application --- -/--	1-18

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

14 December 1994

Date of mailing of the international search report

- 4. 01. 95

 Name and mailing address of the ISA
 European Patent Office, P.B. 5818 Patentaan 2
 NL - 2280 HV Rijswijk
 Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
 Fax (+ 31-70) 340-3016

Authorized officer

Paisdor, B

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 94/03182

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DE,A,28 14 556 (SANKYO CO., LTD.) 12 October 1978 cited in the application see claims ---	1-18
P,A	WO,A,94 12463 (HCT-HEALTH CARE TRADING LTD.) 9 June 1994 see abstract; claims ---	1-18
P,A	WO,A,94 04484 (CORLAY S.L. & METGROVE LTD.) 3 March 1994 see abstract; claims -----	1-18

INTERNATIONAL SEARCH REPORT

Information on patent family members

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. J. Appl. Application No
PCT/EP 94/03182

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		JP-B- 60034540	09-08-85
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		FR-A, B 2395256	19-01-79
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		EP-A- 0609415	10-08-94

(19) World Intellectual Property Organization
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GmbH, Elisenhof, Elisenstrasse 3, 80335 München (DE).

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[DE/DE]; Frankfurter Ring 193a, 80807 München (DE).

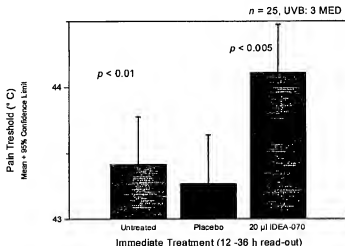
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(72) Inventors; and

(75) Inventors/Applicants (for US only): CEVC, Gregor
[DE/DE]; Tassilostrasse 3, 82131 Gauting (DE);
ROTHER, Matthias [DE/DE]; Lärchenstrasse 3, 82166
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(54) Title: EXTENDED SURFACE AGGREGATES IN THE TREATMENT OF SKIN CONDITIONS



(57) Abstract: The invention relates to the use of extended surface aggregates (ESAs) comprising at least one first amphipathic component, which is a basic aggregate-forming component, and at least one second amphipathic component, which decreases aggregate sensitivity to physical stress, including stress created by enforced passage of said ESAs through pores with an average pore diameter at least 50 % smaller than the average diameter of the ESAs before said passage, such that the average ESA diameter change induced by such physical stress is reduced by 10 % or more, compared to the diameter change induced by such stress in a reference system comprising just the first or just the second aggregate component, in the manufacture of a pharmaceutical preparation for enduring treatment of pathological mammalian skin conditions, including skin irritation, skin inflammation and/or skin damage after topical application, for modifying skin pigmentation and/or for treatment of skin itch.

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Extended surface aggregates in the treatment of skin conditions

The invention broadly concerns the application of actives, especially pharmaceutical
5 drug substances, to mammalian, especially human, skin. In one aspect, the invention
concerns the treatment of pathological skin conditions including irritation, pain,
itching, inflammation and/or skin damage. More specifically the invention concerns
the use of extended surface aggregates, including bilayer membranes, based on
amphipathic components, especially lipids, in the manufacture of pharmaceutical
10 preparations for the treatment of such pathological skin conditions.

In another aspect, the invention relates to methods and formulations suitable for
modifying skin pigmentation in living organisms provided with pigmented skin, and
especially in humans and animals. Specifically, the invention is concerned with
15 formulations and methods suitable to induce depigmentation in vivo, without causing
skin damage. The invention is also concerned with methods of treating diseases
related to hyperpigmentation and pigment cell proliferation.

The skin, including the skin of all mammals, has evolved to become one of the best
20 biological barriers known to mankind. This barrier function is required both to keep
necessary substances from leaving the body, and to keep undesired substances from
entering the body.

In mammals, this barrier function of the skin is mainly provided by the outermost
25 horny layer of the skin, the stratum corneum.

Many attempts have been made in the past to find transdermal formulations, capable
of transporting actives (e.g. pharmaceutical agents) to their destined location in the
body (e.g. in muscle tissue or organs) through the intact skin. Generally, such early
30 attempts have been insufficiently effective.

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- A major breakthrough in transdermal therapy was achieved when it was found that specific mixed lipid bilayers with high permeability and high flexibility characteristics are capable of overcoming narrow, normally confining pores. Often, these take the form of extremely deformable vesicles enclosed by a (generally single) bilayer membrane. The bilayers are formed from amphipathic substances e.g. phosphatidylcholine, which typically form liposomes. Their flexibility is provided by admixture of membrane softening compounds, e.g. surfactants. Vesicles provided with such mixed lipid bilayer membranes can permeate through passages in the skin which would otherwise not even permit the penetration of their constituent molecules. It is assumed that this is based on the opening of initially very narrow (0.4 nm) intercellular hydrophilic channels in the stratum corneum lipid layer by these vesicles, to form hydrophilic pores approx. 20 nm wide, through which the ultradeformable vesicles can then permeate.
- 15 This technology is protected by a series of granted patents and patent applications. An early example is EP 0 475 160. A more recent example is WO 2004/032900. A recent scientific article explaining this technology is G. Cevc, A. G. Schätzlein, H. Richardsen and U. Vierl, "Overcoming semi permeable barriers, such as the skin, with ultradeformable mixed lipid vesicles, transfersomes, liposomes or mixed lipid micelles", *Langmuir* 2003, 19, 10753-10763. In the literature, vesicles incorporating this technology are often indicated using a trademark owned by the instant applicant, comprising the term "transfersome". In the context of this description, the term "transfersome" will be used to designate an ultra-deformable vesicle incorporating this technology, as described in the above-mentioned references and commercially available from the applicant. More generally, highly deformable mixed lipid bilayers (whether vesicular or not) will be referred to as "Extended Surface Aggregates" or ESA's.
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- 25

The published literature describes the use of transfersomes for the transport of actives through the skin, to that part of the body, where their pharmaceutical activity is required. Especially the older transfersome literature stresses the fact that transfersome vesicles penetrate the skin intact, i.e. with the active ingredient carried (as associated with the transfersome material) not only into, but also through and out of the (widened) pores in the stratum corneum, through the underlying epidermal strata and through the dermis, without destruction of the vesicle (although some average size reduction may, in case, be observed). In the treatment of body parts interior of the dermis, this is necessary, to avoid the active being carried off by the blood circulation system, before the destined locus is reached.

Summary of the invention

The present invention is based on the concept of using such mixed lipid bilayer structures or extended surface aggregates, (ESA's) as generally described in the above-mentioned literature (especially in WO 2004/032900) for the treatment of the skin itself, where a skin condition in need of such treatment exists.

Pathological skin conditions do not necessarily involve major structural changes in the skin, and specifically do not generally involve the loss of the stratum corneum's barrier function. Indeed, the pathological skin conditions on which the present invention is mainly focused, leave the barrier function of the stratum corneum basically intact.

Typical such pathological skin conditions include skin irritation, pain, itching, inflammation and/or skin damage, without concurrent loss of the skin's barrier function. Thus, while the skin is not in its natural condition, the skin barrier is functioning. Typical examples include sunburn and other forms of dermatitis.

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The skin condition may alternatively have been caused by a treatment that at least partly removes the outer skin cell layers, e.g. erosive laser treatments as used for therapeutic and cosmetic purposes.

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The skin condition may be caused by exposure to chemicals, especially skin irritants. The invention e.g. includes the use of ESA's in the therapy of allergies, such as contact allergies.

- 10 Generally, reference to therapeutical uses herein is to be understood to include, besides therapy of already existing pathological conditions, also the prevention of such conditions.

- 15 In another aspect, the invention concerns the modification of skin pigmentation. It is known that the changes in skin pigmentation can be induced by pharmaceutically active substances.

- Skin pigmentation can for example be increased by stimulation of melanocytes, and this may be caused by the application of drugs like cyclophosphamid, MTX, 5-FU, 20 chlofazimin, phenotiazine, thiazide, tetracycline and also NSAIDs (i.e. Non-Steroidal Anti-Inflammatory Drugs).

- Depigmentation or hypopigmentation, i.e. the decrease of the concentration of pigments in the skin, can be caused by skin damage (e.g. drug eruptions, contact 25 dermatitis, scarring) induced by various pharmaceutically active substances, including NSAIDs.

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In an article by Zailaie, Saudi Med J. 2004 Nov.; 25 (11): 1656-63, in-vitro studies in cell cultures are reported, which appeared to show that in such cell cultures, low concentrations of acetylsalicylic acid stimulate melanocytes, whereas very high concentrations may cause melanocyte apoptosis.

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To the Applicant's knowledge, it has not yet been reported that actives such as NSAIDs can induce depigmentation in vivo, in human or animal skin that has not initially been damaged by the drug.

- 10 It has now surprisingly been found in the context of a clinical trial, as described below, that transfersome preparations of NSAIDs as described herein can induce profound depigmentation (or hypopigmentation) in vivo, in the absence of any skin damage. Without wishing to be bound to any theory, it is presently assumed that the unparalleled efficacy of transfersomes (and other such amphipathic aggregates, as
- 15 e.g. described in US 10/357 617) in transporting actives through the stratum corneum, to (and beyond) the deeper strata of the skin, creates exposure of the melanocytes to such high local concentrations of active, that impairment of melanocyte function or even apoptosis can be induced.

- 20 Formulations suitable for providing this depigmentation effect include the ones described in above-mentioned US patent application serial-no. 10/357 617.

Methods of treatment in accordance with this invention include the application of such formulations onto the skin to be treated for extended time periods, up to several

25 days or even weeks, as found necessary.

This invention is useful where treatment of hyperpigmentation or melanocyte dysfunction is desired.

Another potential use of the invention is in the treatment of undesired pigmentation. It is expected that by suitably selecting the pharmaceutically active substance, by selecting its concentration in the formulation and by selecting the time period of treatment, very different effects can be achieved, ranging from a persistent general hypopigmentation, which might just meet cosmetical needs, through the treatment of melasma and melanoma. It is expected that at suitably high active concentrations and suitably long treatment, apoptosis (cell-death) of melanocytes exposed to the treatment can be induced, so that it is possible that undesired growth of melanocytes can be reduced, or noxious melanocyte populations may indeed be entirely removed, which could provide a treatment for e.g. melanoma.

Definitions

In the present invention, the general terms employed hereinbefore and hereinafter have the following meanings.

The term "**active**" means a pharmaceutical active or drug.

The term "**aggregate**" denotes a group of more than just a few amphipaths of similar or different kind. Typically, an aggregate referred to in this invention contains at least 100 molecules, i.e. has an aggregation number $n_a > 100$. More often aggregation number is $n_a > 1000$ and most preferably $n_a > 10.000$. An aggregate comprising an aqueous core surrounded with at least one lipid (bilayer) membrane is called a lipid vesicle, and often a liposome.

The term aggregate "**adaptability**" is defined in this document as the ability of a given aggregate to change easily and more or less reversibly its properties, such as

- shape, elongation ratio, and surface to volume ratio. Adaptability also implies that an aggregate can sustain unidirectional force or stress, such as a hydrostatic pressure, without significant fragmentation, as is defined for the "stable" aggregates. An easy and reversible change in aggregate shape furthermore implies high aggregate
- 5 deformability and requires large surface-to-volume ratio adaptation. For vesicular aggregates, the latter is associated with material exchange between the outer and inner vesicle volume, i.e. with at least transient vesicle membrane permeabilisation. The experimentally determined capability of given aggregate suspension to pass through narrow pores in a semi-permeable barrier thus offers simple means for
- 10 functionally testing aggregate adaptability and deformability (*vide supra*), as is described in the Practical Examples.

To assess aggregate adaptability it is useful to employ the following method:

- 15 1) measure flux j_a of aggregate suspension through a semi-permeable barrier (e.g. gravimetrically) for different transport-driving trans-barrier pressures Δp ;
- 2) calculate the pressure dependence of barrier penetrability P for given suspension by dividing each measured flux value with the corresponding driving pressure value: $P(\Delta p) = j_a(\Delta p) / \Delta p$;
- 20 3) monitor the ratio of final and starting vesicle diameter $2r_{ves}(\Delta p)/2r_{ves,0}$ (e.g. with the dynamic light scattering), wherein $2r_{ves}(\Delta p)$ is the vesicle diameter after semi-permeable barrier passage driven by Δp and $2r_{ves,0}$ is the starting vesicle diameter, and if necessary making corrections for the flow-rate effects;
- 25 4) align both data sets $P(\Delta p)$ vs. $r_{ves}(\Delta p)/r_{ves,0}$, to determine the co-existence range for high aggregate adaptability and stability; it is also useful, but not absolutely essential, to parameterise experimental penetrability data within the framework of Maxwell-approximation in terms of the necessary

pressure value p^* and of maximum penetrability value P_{max} , which are defined graphically in the following illustrative schemes.

- It is plausible to sum up all the contributions to a moving aggregate energy (deformation energy/ies, thermal energy, the shearing work, etc.) into a single, total energy. The equilibrium population density of aggregate's energetic levels then may be taken to correspond to Maxwell's distribution, All aggregates with a total energy greater than the activation energy, $E_f E_A$, are finally concluded to penetrate the barrier. The pore-crossing probability for such aggregates is then given by:

10

$$P(e) = 1 - \operatorname{erf}\left(\sqrt{\frac{1}{e}}\right) + \sqrt{\frac{4}{\pi e}} \cdot \exp\left[-\frac{1}{e}\right],$$

e being dimensionless aggregate energy in units of the activation energy E_A .

- 15 It is therefore plausible to write barrier penetrability to a given suspension as a function of transport driving pressure (= driving pressure difference) p ($=\Delta p$) as:

$$P(p) = P_{max} \cdot \left\{ 1 - \operatorname{erf}\left(\sqrt{\frac{p^*}{p}}\right) + \sqrt{\frac{4p^*}{\pi p}} \cdot \exp\left[-\frac{p^*}{p}\right] \right\} \quad (*)$$

- 20 P_{max} is the maximum possible penetrability of a given barrier. (For the aggregates with zero transport resistance this penetrability is identical to the penetrability of the suspending medium flux.) p^* is an adjustable parameter that describes the pressure sensitivity, and thus the transport resistance, of the tested system. (For barriers with a fixed pore radius this sensitivity is a function of
- 25 aggregate properties solely. For non-interacting particles the sensitivity is

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dominated by aggregate adaptability, allowing to make the assumption: a_d proportional to $1/p^*$.)

The formula (*) is used to calculate aggregate adaptability from suspension flux, or more precisely from the corresponding penetrability ($= P(p) = \text{Flux} / \text{Pressure} = \text{Flux} / p$ data).

This formula is explained, in more detail, in our co-pending U.S. application Serial No.: 10/357 618 "Aggregates with increased deformability, comprising at least three amphipaths, for improved transport through semi-permeable barriers and for the non-invasive drug application in vivo, especially through the skin", the disclosure of which is incorporated herein by reference.

The term "**apparent dissociation constant**" refers to the measured dissociation (i.e. ionisation) constant of a drug. This constant for many drugs, including NSAIDs, is different in the bulk and in the homo- or heteroaggregates. For ketoprofen, the pKa in the bulk is approx. 4.4 whereas the pKa value measured above the drug association concentration is approx. 5, and decreases approximately linearly with the inverse ionic strength of the bulk solution. pKa of ketoprofen bound to lipid bilayers increases with total lipid concentration as well, and is approx. 6 and 6.45 in suspensions with 5 w-% and 16 w-% total lipid in a 50 mM monovalent buffer, respectively. For diclofenac, the pKa in the bulk is around 4, whereas for this drug in lipid bilayers pKa ~ 6.1 was determined. The bulk pKa reported in the literature for meloxicam, piroxicam, naproxen, indomethacin and ibuprofen is 4.2 (and 1.9), 5.3, 4.2-4.7, 4.5, and 4.3 (or in some reports 5.3), respectively.

The term aggregate "**deformability**" is closely related to the term "adaptability". Any major change in aggregate shape that does not result in a significant aggregate fragmentation is indicative of sufficient aggregate deformability, and also implies a large

- 10 -

change in the deformed aggregate surface-to-volume ratio. Deformability can therefore be measured in the same kind of experiments as is proposed for determining aggregate adaptability, or else can be assessed by optical measurements that reveal reversible shape changes.

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The term "**narrow**" used in connection with a pore implies that the pore diameter is significantly, typically at least 30%, smaller than the diameter of the entity tested with regard to its ability to cross the pore.

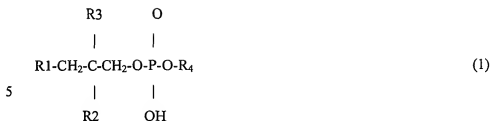
- 10 The term "**NSAID**" (non-steroidal anti-inflammatory drug) typically indicates a chemical entity which acts as cyclooxygenase-1 and/or cyclooxygenase-2 antagonist. Within the framework of this invention lipoxigenase inhibitors are also considered to be part of the class of NSAID's.

- 15 Examples include salts of substituted phenylacetic acids or 2-phenylpropionic acids, such as alclofenac, ibufenac, ibuprofen, clindanac, fenclo rac, ketoprofen, fenoprofen, indoprofen, fenclofenac, diclofenac, flurbiprofen, pirofen, naproxen, benoxaprofen, carprofen or cicloprofen; analgesically active heteroarylacetic acids or 2-heteroarylpropionic acids having a 2-indol-3-yl or pyrrol-2-yl radical, for example
20 indomethacin, oxmetacin, intrazol, acetamin, cinmetacin, zomepirac, tolmetin, colpirac or tiaprofenic acid; analgesically active indenylacetic acids, for example sulindac; analgesically active heteroarylloxyacetic acids, for example benzadac; NSAIDS from the oxicam family include piroxicam, droxicam, meloxicam, tenoxicam; further interesting drugs from NSAID class are, meclofenamate, etc.

25

The term "**phospholipid**" means, for example, compounds corresponding to the formula

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in which one of the radicals R1 and R2 represents hydrogen, hydroxy or C1-C4-alkyl, and the other radical represents a long fatty chain, especially an alkyl, alkenyl, alkoxy, alkenyloxy or acyloxy, each having from 10 to 24 carbon atoms, or both radicals R1 and R2 represent a long fatty chain, especially an alkyl, alkenyl, alkoxy, alkenyloxy or acyloxy each having from 10 to 24 carbon atoms, R3 represents hydrogen or C1-C4-alkyl, and R4 represents hydrogen, optionally substituted C1-C7-alkyl or a carbohydrate radical having from 5 to 12 carbon atoms or, if both radicals R1 and R2 represent hydrogen or hydroxy, R4 represents a steroid radical, or is a salt thereof. The radicals R1, R2, R3, and R4 are typically selected so as to ensure that lipid bilayer membrane is in the fluid lamellar phase during practical application and is a good match to the drug of choice.

In a phospholipid of the formula 1, R1, R2 or R3 having the meaning C1-C4-alkyl is preferably methyl, but may also be ethyl, n-propyl, or n-butyl.

The terms alkyl, alkenyl, alkoxy, alkenyloxy or acyloxy have their usual meaning, expressed in detail in parallel patent application. The long fatty chains attached to a phospholipid can also be substituted in any of usual ways.

A steroid radical R4 is, for example, a sterol radical that is esterified by the phosphatidyl group by way of the hydroxy group located in the 3-position of the

steroid nucleus. If R4 represents a steroid radical, R1 and R2 are preferably hydroxy and R3 is hydrogen.

Phospholipids of the formula I can be in the form of free acids or in the form of salts.

- 5 Salts are formed by reaction of the free acid of the formula II with a base, for example a dilute, aqueous solution of alkali metal hydroxide, for example lithium, sodium or potassium hydroxide, magnesium or calcium hydroxide, a dilute aqueous ammonia solution or an aqueous solution of an amine, for example a mono-, di- or tri-lower alkylamine, for example ethyl-, diethyl- or triethyl-amine, 2-hydroxyethyl-
10 tri-C1-C4-alkyl-amine, for example choline, and a basic amino acid, for example lysine or arginine.

- A phospholipid of the formula I has especially two acyloxy radicals R1 and R2, for example alkanoyloxy or alkenoyloxy, for example lauroyloxy, myristoyloxy,
15 palmitoyloxy, stearoyloxy, arachinoyloxy, oleoyloxy, linoyloxy or linoleoyloxy, and is, for example, natural lecithin (R3 = hydrogen, R4 = 2-trimethylammonium ethyl) or cephalin (R3 = hydrogen, R4 = 2-ammonium ethyl) having different acyloxy radicals R1 and R2, for example egg lecithin or egg cephalin or lecithin or cephalin from soya beans, synthetic lecithin or cephalin having different or identical acyloxy
20 radicals R1 and R2, for Example 1-palmitoyl-2-oleoyl lecithin or cephalin or dipalmitoyl, distearoyl, diarachinoyl, dioleoyl, dilinoyl or dilinoleoyl lecithin or cephalin, natural phosphatidyl serine (R3 = hydrogen, R4 = 2-amino-2-carboxyethyl) having different acyloxy radicals R1 and R2, for example phosphatidyl serine from bovine brain, synthetic phosphatidylserine having different or identical acyloxy
25 radicals R1 and R2, for example dioleoyl-, dimyristoyl- or dipalmitoyl-phosphatidyl serine, or natural phosphatidic acid (R3 and R4 = hydrogen) having different acyloxy radicals R1 and R2.

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- A phospholipid of the formula 1 is also a phospholipid in which R1 and R2 represent two identical alkoxy radicals, for example n-tetradecyloxy or n-hexadecyloxy (synthetic ditetradecyl or dihexadecyl lecithin or cephalin), R1 represents alkenyl and R2 represents acyloxy, for example myristoyloxy or palmitoyloxy (plasmalogen, R3 = hydrogen, R4 = 2-trimethylammonium ethyl), R1 represents acyloxy and R2 represents hydroxy (natural or synthetic lysolecithin or lysocephalin, for Example 1-myristoyl- or 1-palmitoyl-lyso-lecithin or -cephalin; natural or synthetic lysophosphatidyl serine, R3 = hydrogen, R4 = 2-amino-2-carboxyethyl, for example lysophosphatidyl serine from bovine brain or 1-myristoyl- or 1-palmitoyl-lysophosphatidyl serine, synthetic lysophosphatidyl glycerine, R3 = hydrogen, R4 = CH₂OH-CHOH-CH₂-, natural or synthetic lysophosphatidic acid, R3 = hydrogen, R4 = hydrogen, for example egg lysophosphatidic acid or 1-lauroyl-, 1-myristoyl- or 1-palmitoyl-lysophosphatidic acid).
- 15 The term "**semipermeable**" used in connection with a barrier implies that a suspension can cross transbarrier openings whereas a suspension of non-adaptable aggregates 150-200% larger than the diameter of such openings cannot achieve this. Conventional lipid vesicles (liposomes) made from any common phospholipid in the gel lamellar phase or else from any biological phosphatidylcholine/cholesterol 1/1
- 20 mol/mol mixture or else comparably large oil droplets, all having the specified relative diameter, are three examples for such non-adaptable aggregates.
- The terms "**stable**" and "**sufficiently stable**" mean that the tested aggregate does not change its diameter spontaneously or under relevant mechanical stress (e.g. during
- 25 passage through a semipermeable barrier) to a practically (most often: pharmaceutically) unacceptable degree. A 20-40 % change is considered acceptable; the halving of aggregate diameter or a 100 % diameter increase is not.

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The term "**sterol radical**" means, for example, the lanosterol, sitosterol, coprostanol, cholestanol, glycocholic acid, ergosterol or stigmasterol radical, is preferably the cholesterol radical, but can also be any other sterol radical known in the art.

- 5 The term "**surfactant**" also has its usual meaning. A long list of relevant surfactants and surfactant related definitions is given in EP 0 475 160 and US 6 165 500 which are herewith explicitly included by reference and in appropriate surfactant or pharmaceutical Handbooks, such as *Handbook of Industrial Surfactants* or US Pharmacopoeia, Pharm. Eu., etc. Surfactants are typically chosen to be in a fluid
- 10 chain state or at least to be compatible with the maintenance of fluid-chain state in carrier aggregates.

- The term "**surfactant like phospholipid**" means a phospholipid with solubility, and other relevant properties, similar to those of the corresponding surfactants mentioned
- 15 in this application, especially in the claims 10 and 11. A non-ionic surfactant like phospholipid therefore should have water solubility, and ideally also water diffusion / exchange rates, etc., similar to those of a relevant non-ionic surfactant.

Detailed description of the invention

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- In the context of this description, the invention will be exemplified in the context of skin analgesia and inflammation, in the context of skin pigmentation, and in treating itch. It is to be understood, however, that the invention is not limited to such treatments, and in fact extends to all preventive and therapeutical treatments of the
- 25 skin, especially the human skin, which involve correspondingly usable pharmaceutical actives.

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In the preferred embodiments, the use of NSAIDs is exemplified. NSAIDs are a preferred class of drugs for practising this invention. It should be understood, however, that other classes of drugs can as well be used in similar treatments of pathological skin conditions. The invention is also not limited to analgesic applications, but extends to the treatment of all kinds of pathological conditions of the mammalian skin.

NSAIDs ("non-steroidal anti-inflammatory drugs") are a class of drugs with many very well known members. A definition is provided below in the "Definitions" section.

The only currently marketed NSAID formulation in the US for the treatment of any pathological skin condition (Solaraze ®) is a diclofenac product for use in actinic ceratosis (praecancerous). This product is reported to cause skin irritation in up to 60 % of the treated patients, and seems to be unacceptable for use in inflamed skin conditions.

Sunburn is a model of skin inflammation and a major source of skin pain experienced by humans. It is a clinical response to acute cutaneous solar photo damage after an excessive exposure to ultraviolet, especially UVB light and ranges from mild, painless cutaneous erythema to painful erythematous skin with associated oedema and blistering. There are no standard treatments for sunburn. A combination of non-pharmacological and pharmacological treatment modalities is currently used to treat sunburn, including topical application of hydrocortisone, but none of these current therapies is considered to be sufficiently efficient.

It is basically known that painful, inflammatory skin conditions such as sunburn and other types of dermatitis, react to the use of NSAIDs, such as indomethacin (Khidbey

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and Kurban, Journal of Investigative Dermatology 66, 153-156 (1976); Farr and Diffey, British Journal of Dermatology (1986) 115, 453-456; Juhlin and Shroot, Acta derm. Venereol. (Stockh 1992); 72: 222-223). Herein, indomethacin was used in a gel base or in alcoholic solution, and found to provide some inhibition of the appearance of erythema.

Presently, no NSAID formulation is however approved for the treatment of any painful, inflammatory skin condition. In fact, NSAID formulations are contraindicated for the use on irritated and pre-damaged skin. While NSAIDs such as indomethacin may be (limitedly) effective, the irritation potential of corresponding preparations basically prevents use on irritated and predamaged skin.

Besides sunburn there are several comparable painful and often inflammatory skin conditions, which might benefit from anti inflammatory and analgesic treatments. Besides other forms of dermatitis, these include itching, skin damage and skin irritations caused by treatments such as laser therapy.

However (on top of their irritative properties), the known topical formulations are not sufficiently efficient. In the absence of penetration enhancers, such as alcohol, hardly any active actually passes the stratum comeum, which prevents the required pharmaceutical effect. The use of penetration enhancers, especially alcohol, is in itself detrimental where the skin is irritated or damaged, since the use of penetration enhancers then often leads to increased irritation. Even in the presence of penetration enhances, the actives do not penetrate the stratum comeum in sufficient concentrations, to provide the required pharmaceutical efficacy.

Mechanical and electrical methods for providing enhanced transdermal efficiency (iontophoresis, electroporation etc.) are generally unsuitable, because they again increase irritation and pain, where the skin is already irritated and/or damaged.

- 5 A need therefore exists for pharmaceutical preparations for the treatment of pathological mammalian skin conditions, which may include skin irritation, skin inflammation and/or skin damage, which makes it possible to transport suitable pharmaceutical actives to their desired locus of activity, and which provides efficient transport of the pharmaceutical active through the stratum corneum, especially
- 10 without the irritative side-effects of the known preparations.

- One object of the invention is therefore to provide pharmaceutical preparations, which may provide a higher efficacy of active penetration through the stratum corneum, for the treatment of pathological mammalian skin conditions, including but
- 15 not limited to inflammatory conditions, dermatitis, skin irritation, pain, hyperpigmentation and pigment cell proliferation, and itching.

- Another important object of the invention is to provide such pharmaceutical preparations which are safe to be used on irritated and/or pre-damaged skin.

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Yet another object of the invention is to provide such pharmaceutical preparations which can carry a sufficient drug load through the stratum corneum into the dermis.

- In another aspect, the objectives of the invention comprise the provision of new or
- 25 improved treatments for the above-outlined undesired skin conditions.

In one major aspect of the invention, these objectives are attained by the use of extended surface aggregates (ESAs) comprising at least one first amphipathic

component which is a membrane forming lipid component and at least one second amphipathic component which is a membrane destabilising component, whereby the ESA is also capable of penetrating semi-permeable barriers with pores, the greatest diameter of said pores being at least 50 % smaller than the average diameter of the

5 ESAs before the penetration, without changing the average ESA diameter by more than 25 %, in the manufacture of a pharmaceutical preparation for the treatment of pathological mammalian skin conditions including skin irritation, skin inflammation and/or skin damage.

- 10 In a preferred embodiment of the invention, the ESAs comprise at least one third amphipathic component which is also a membrane destabilising component.

In a highly preferred embodiment of the invention, one membrane destabilising component in the extended surface aggregate is itself an active, especially a non-

15 steroidal anti-inflammatory drug (NSAID).

The penetration capability of the ESAs is evaluated using semi-permeable barriers with pores, typically formed by synthetic membranes with known, sufficiently homogenous pore diameters.

- 20 The use of such semi-permeable synthetic membranes as a barrier model is described in the art, e.g. in the above mentioned article by Cevc et al. in *Langmuir*, Volume 19, Number 26, Pages 10753-10763. Such membranes preferably have pore diameters around 20 nm, since this corresponds to the pore size in mammalian skin when the
- 25 hydrophilic skin pores are widened by the permeation of the inventive extended surface aggregates (ESAs), especially transfersomes.

- 19 -

Generally speaking, ESAs suitable for practicing this invention are known in the art, for different applications. Specifically, such ESAs are described in WO 2004/032900, as above mentioned, the complete contents whereof are therefore hereby incorporated by reference. Some parts of the disclosure of WO 2004/032900 are recited below.

The main difference between this art and the invention lies in the fact that in the reference, the specific use of ESAs to treat pathological mammalian skin conditions is not disclosed, and the preferred parameters which render this use most effective, are not specifically disclosed either. These parameters specifically include the preferred area doses, which differ in the inventive dermatological applications, from the area doses required for transdermal applications in deeper body tissues, such as muscle. The applied area doses suitable for practicing this invention vary, depending on the active used.

One highly preferred active for practicing the present invention is ketoprofen. Ketoprofen is especially preferred, since it is both a Cox 1 and Cox 2 inhibitor and inhibits lipoxxygenase activity, so that it can reduce prostaglandin and leucotriene mediated inflammatory reactions.

Typical applied area doses for ketoprofen on human skin are above 0.005 mg per cm² of skin area, more preferably above 0.01 mg and even more preferably lie at 0.02 mg per cm² of skin area or above.

Typically, the applied area dose will not exceed 1 mg per cm², more preferably 0.5 mg per cm² and even more preferred, not more than 0.25 mg per cm².

- 20 -

In presently preferred embodiments, the applied area dose is between 0.01 and 0.07 mg, even more preferred between 0.02 and 0.06 mg ketoprofen per cm² of human skin. 0.06 mg / cm² is a highly preferred applied area dose.

- 5 Similar applied area doses may be used for diclofenac, flurbiprofen, piroxicam and other oxicam actives such as meloxicam, tenoxicam etc., as well as other actives with a potency comparable to ketoprofen.

- 10 Applied area doses for other NSAIDs, including indomethacin, ketorolac, ibuprofen and naproxen, would be higher, preferably up to and including 10 times higher than the above values given for ketoprofen. For other actives such as salicylates, pyrazalone derivatives (phenylbutazone etc.) or tolmetine, applied area doses would be even higher, up to and including 100 times the above given range for ketoprofen.

- 15 The formulations used will generally be as little skin irritating as possible. The ESAs used in accordance with this invention are by definition provided with transdermal activity, which involves the widening of skin pores and therefore some active interference with the epidermis. They generally do not need added penetration enhancers in order to perform. It is therefore possible, and also desirable, to keep the
20 use, and respective concentration, of chemical skin irritants as components of these systems, as low as possible. Thus, formulations using e.g. very little alcohol or no alcohol (especially ethanol) as possible, may be beneficial.

- The same applies with respective other potential skin irritants.

25

The relatively small applied area does of this invention assist in avoiding skin irritation caused by the pharmaceutical preparation. The preferred use of low dosage formulations such as spray formulations contributes to irritation avoidance.

- 21 -

Quite detailed recommendations on the preparation of inventive combinations are given in EP 0 475 160 and US 6 165 500, which are herewith included by reference, using filtering material with pore diameters between 0.01 μm and 0.1 μm , more
5 preferably with pore diameters between 0.02 μm and 0.3 μm and even more advisable filters with pore diameters between 0.05 μm and 0.15 μm to homogenise final vesicle suspension, when filtration is used for the purpose. Other methods of mechanical homogenisation or for lipid vesicle preparation known in the art are useful as well.

10 The lipids and certain surfactants mentioned hereinbefore and hereinafter having a chiral carbon atom can be present both in the form of racemic mixtures and in the form of optically pure enantiomers in the pharmaceutical compositions that can be prepared and used according to the invention.

15 To manufacture a pharmaceutical formulation, it may advisable or necessary to prepare the product in several steps, changing temperature, pH, ion strength, individual component (e.g. membrane destabiliser, formulation stabiliser or microbicide) or total lipid concentration, or suspension viscosity during the process.

20 A list of relevant and practically useful thickening agents is given e.g. in PCT/EP98/08421, which also suggests numerous interesting microbicides and antioxidants; the corresponding sections of PCT/EP98/08421 are therefore included into the present application by reference. Practical experiments have confirmed that sulphites, such as sodium sulphite, potassium sulphite, bisulphite and metasilphite; and potentially
25 other water soluble antioxidants, which also contain a sulphur or else a phosphorus atom (e.g. in pyrosulphate, pyrophosphate, polyphosphate), erythorbate, tartrate, glutamate, etc. or even L-tryptophan), ideally with a spectrum of activity similar to that of sulphites) offer

some anti-oxidative protection to said formulations, final selection being subject to regulatory constraints. Any hydrophilic antioxidant should always be combined with a lipophilic antioxidant, however, such as BHT (butylated hydroxytoluene) or BHA (butylated hydroxyanisole).

5

Embodiment Examples

The invention will now be illustrated in more detail, based on the following examples.

10 Example 1

In a first embodiment example, a ketoprofen formulation for the topical treatment of painful skin conditions according to the invention is composed as in **Table 1**:

15

- 23 -

Compound	Function	Concentration (mg/g)
Ketoprofen, EP	Active agent	23.82
Soy phosphatidylcholine (SPC)	Carrier agent	71.46
Ethanol 96 %, EP	Solvent	35.00
Polysorbate 80, EP	Carrier agent	4.72
Sodium hydroxide, EP	Base	4.10
Disodium phosphate dodecahydrate, EP	Buffering agent	16.39
Sodium dihydrogen phosphate dihydrate, EP	Buffering agent	0.66
Sodium metabisulphite, EP	Antioxidant	0.50
Disodium edetate, EP	Chelator	3.00
Butylhydroxyanisole, EP	Antioxidant	0.20
Methyl parahydroxybenzoate, EP	Preservative	2.50
Ethyl parahydroxybenzoate, EP	Preservative	1.70
Propyl parahydroxybenzoate, EP	Preservative	0.50
Linalool, FCC	Odor masking agent	1.00
<i>Benzyl alcohol, EP (optional)</i>	Preservative and stabiliser	5.25
Glycerol 85%, EP	Humectant	50.00
Water, purified, EP	Solvent	779.20
Total		1000.00

Table 1

Example 2

5

It will be noted that the composition of Example 1 comprises relevant amounts of lower aliphatic alcohol (ethanol) which may irritate the skin. A presently more preferred embodiment, comprising no ethanol, is shown in **Table 2**:

10

- 24 -

Compound	Function	Concentration (mg/g)
Ketoprofen, EP	Active agent	4.76
Soy phosphatidylcholine (SPC)	Carrier agent	14.30
Polysorbate 80, EP	Carrier agent	0.94
Sodium hydroxide, EP	Base	0.70
Disodium phosphate dodecahydrate, EP	Buffering agent	8.20
Sodium dihydrogen phosphate dihydrate, EP	Buffering agent	0.33
Sodium metabisulphite, EP	Antioxidant	0.30
Disodium edetate, EP	Chelator	1.00
Butylhydroxyanisole, EP	Antioxidant	0.08
Propyl parahydroxybenzoate, EP	Preservative	1.00
<i>Butyl parahydroxybenzoate, EP (optional)</i>	<i>Preservative</i>	<i>1.00</i>
Linalool, FCC	Odor masking agent	0.50
Glycerol 85%, EP	Humectant	20.00
Water, purified, EP	Solvent	946.89
Total		1000.00

Table 2

Example 3

5

Another preferred embodiment, with a small ethanol content, has the following composition:

- 25 -

Compound	Concentration (mg/g)
SPC S100	14.30
Ketoprofen	4.76
Tween 80	0.94
Ethanol	3.00
Glycerol	20.00
Imidazolidinyl urea	2.50
BHA	0.04
Na-Metabisulfite	0.25
EDTA	3.00
Linalool	0.20
Na ₂ HPO ₄ x 12 H ₂ O	8.34
NaH ₂ PO ₄ x 2 H ₂ O	0.27
NaOH	1.13
Water, purified, EP	941.27
Total	1000.00

Total lipid concentration is 2 wt%. Active content (Ketoprofen) is 0.476 wt%. The final product has a pH of 7.9.

5

Example 4

A clinical trial was carried out, to study the effect of inventive treatments, on pathological skin conditions including pain and inflammation.

10

The preparation used was as described in Example 1 above.

The study had a randomised, double-blind, placebo and active controlled format. The primary objective was to compare the effects of a pharmaceutical preparation in

accordance with this invention, with placebo, on UVB-skin inflammation. The study involved 25 volunteers.

The study included healthy volunteers of skin type II according to Fitzpatrick, aged 18 – 45 years. All subjects were non-smokers or infrequent smokers (less than 5 cigarettes per day) and willing not to smoke at least one hour before the procedure started. Exclusion criteria comprised sun tanning four weeks prior to study; pregnancy or lactation; dermal and systemic diseases; mental disorders; any other chronic or acute illness requiring treatment, including dysplastic naevi and praecancerosis. Exclusion criteria further comprised subjects who had used immunosuppressants (e.g. corticosteroids) within two weeks prior to the study, or had a known sensitivity to NSAIDs, a known photo-allergie/light dermatosis, and substance abusers. The measure of the study was the effect on threshold to heat-induced local pain and erythema following specified UVB irradiation.

Further objectives included the comparison with an equal volume of a commercial product containing hydrocortisone-21-acetate (HC), as well as the testing of lower doses of the inventive preparation, and an evaluation of different application regimes – either immediately after UVB irradiation, or with a delay in treatment.

A comparison was made between skin areas receiving no treatment and no irradiation (control); areas receiving 20 µl of the formulation describes in Example 1 above; areas receiving 20 µl placebo, and areas receiving 20 µl of 0.25 wt % solution of hydrocortisone-21-acetate.

While some skin areas received their treatment directly after UVB irradiation another group received their treatment six hours after UVB irradiation.

- 27 -

In a dose finding part of the study, the amount of formulation according to Example 1 above was varied between 20 μ l, 10 μ l and 5 μ l.

Pain threshold was evaluated in degrees centigrade, erythema and oedema were
5 evaluated on a subjective categorical scale from 0 to 4.

In evaluating the study's primary objective, the effect of 20 μ l of a preparation according to Example 1 above was compared to placebo on subjects with UVB-induced sunburn and corresponding induced hyperalgesia to heat.
10

Figure 1 shows the result for treatment directly after UVB irradiation (3 MED). At 12-36 h read-out, the inventive treatment shows a statistically significant effect over control and placebo.

15 Figure 2 shows the effect of 20 μ l of the Example 1 formulation, on UVB (sunburn) induced hyperalgesia, again for treatment immediately after UVB exposure and at 12-36 h read-out, this time compared to the effect of 20 μ l hydrocortisone-21-acetate solution. The effect provided by the invention, as compared to hydrocortisone, is statistically significant superior.

20 Figures 3, 4 and 5 show the results of dose-finding part of the study, again based on the formulation of Example 1, for immediate treatment (3 MED) and read-out at 12-36 h.

25 Figure 5 compares applied doses of 5 μ l, 10 μ l and 20 μ l of the inventive formulation, to, on the one hand, placebo and, on the other hand, 20 μ l of 0,25 wt % hydrocortisone solution.

- 28 -

Figure 3 shows the effect on pain threshold. All three doses tested are significantly superior to placebo and hydrocortisone; there is no relevant effect of dose variation within the tested limits. This may be due to a ceiling effect.

- 5 Figure 4 shows the same comparison, this time in terms of the number of patients where the occurrence of erythema was fully or at least substantially suppressed. Again, the superiority of the invention over hydrocortisone and placebo is statistically significant.
- 10 Figure 5 compares the invention to hydrocortisone and placebo, in terms of the average rank erythema scores, and those patients which produced erythema. It can be seen that only the invention produced any relevant improvement. Again, there is no significant relevance of the dose used.
- 15 The next aspect evaluated in the study was the effect of the various compared medications, when applied with delay after radiation exposure. All treatments were applied 6 hours after UVB exposure. Figures 6 and 7 show the results (read-out at 12-36 h).
- 20 Specifically, Figure 6 showed that after delayed application of 20 µl of the formulation of Example 1, compared to placebo and hydrocortisone, a statistical significant positive treatment effect on hyperalgesia was experienced by the patients (UVB: 3 MED), whereas hydrocortisone was not significantly different from placebo and control.
- 25 In Figure 7, the same treatments are compared in terms of average rank erythema scores. Again, an effect of any statistical significance is only provided by the invention, whereas hydrocortisone is ineffective at 6 hours delay of treatment.

Lastly, Figure 8 shows the effect of the invention on oedema development. The number of observations of either oedema or erythema after UVB exposure (3 MED) is given, for read-out at 12-36 hours. All subjects developed either no or minor oedema, the majority of subjects developing no oedema at all, when treated with the inventive formulation.

As the study shows, the invention is comparable to the known hydrocortisone treatment in increasing the heat induced pain threshold, where the medication is applied immediately after UVB exposure. This is specifically shown in comparison to untreated but irradiated controls.

In the clinical more relevant situation where the medication occurs with delay (as shown in the 6 hours after UVB exposure tests), only the invention increases the pain threshold, whereas hydrocortisone is ineffective.

The invention prevents erythema development very effectively, both when used directly after UVB exposure and when used with 6 hours delay after the exposure. In both cases, hydrocortisone is ineffective.

The invention effectively prevents oedema formation.

No evidence of dermal intolerance or other adverse events were noted.

Example 5

Again using basically the formulation of Example 1 above, but at two different concentrations of ketoprofen, a study was carried out on the effect of inventive treatments on contact dermatitis in pigs.

- 30 -

Allergic contact dermatitis was induced in pigs by application of dinitrofluorobenzene on the skin. The resulting contact eczema were evaluated using the criteria in of Table 3:

5

Score	Criteria (max. score = 12)		
	Extent	Intensity	Induration
0	no erythema	no erythema	normal finding
1	barely perceptible eryth.	macules of pinhead size	nodules of pinhead size
2	slight erythema	lentil-sized macules	doughy lentil-size nodules
3	moderate erythema	confluent macules	confluent firm nodules
4	severe erythema	diffuse macules	diffuse hard lesion

Table 3

The effects observed at 24 hours post treatment are notable from Figure 9.

10

At both applied area doses of 120 $\mu\text{g per cm}^2$ and 480 $\mu\text{g per cm}^2$, a significant effect was observed, with the higher dose somewhat more effective than the lower one.

Example 6

15

In another study, the development of ketoprofen skin concentration (ng/mg) with time was studied at two different applied area doses of a ketoprofen formulation, again as shown in Example 1 above.

At an applied area dose of 0,24 mg ketoprofen per cm² of pig skin, the skin concentration was significantly higher initially, falling off to basically the same skin concentration as provided by an applied area doses of 0,06 mg per cm² after 8 hours post application. The comparison is shown in Figure 10.

5

A comparison with orally administered ketoprofen is shown in Table 4. This lists the applied area dose, the applied total dose and the amount of ketoprofen found in various body tissues after application. The amount in the tissue is given in terms of the AUC (area under the curve) value, for the first 24 hours post application.

10

The data in **Table 4** show the significantly higher skin concentration of active as compared to the concentration in subcutaneous fat, or even deeper lying body tissues such as superficial muscle and deep muscle. As expected, the data indicate that oral ketoprofen provides no topical effect in the skin.

15

Product	AUC _{1-24h} [ng x h x mg ⁻¹]			
	Ex. 1	Ex. 1	Ex. 1	oral KT
Applied area dose (KT / cm ²)	0.5 mg	0.24 mg	0.06 mg	n.a.
Applied total KT dose	50 mg	24 mg	6 mg	50 mg
AUC Skin	n.d.	1022	539	n.d.
AUC subcutaneous fat	710	140	104	11
AUC Superficial muscle	299	89	44	7
AUC Deep muscle	267	59	34	9

n.d. not determined due to inavailability of tissue samples

Table 4

5 Example 7

Safety of the inventive preparation was studied in a dermal irritation / corrosion study according to Council Directive 92/69/EEC, Annex, Method B.4 in rabbits, which was performed with the clinical trial formulation. The rabbits were treated topically on upper dorsum twice daily ten hours apart for 42 consecutive days with an area dose of 0.23 mg KT per cm², the same area dose that has also been used in the clinical study. Rabbits were Draize-scored (scores from 0 to 4) twice daily prior to test article application for erythema and oedema, also allowing half-value readings.

15 All animals showed only slight temporary signs of dermal irritation. At the end of the study (day 42) none of the rabbits showed signs of dermal irritation.

Due to the lower drug concentration and overall lower excipient concentrations in formulations as given in Example 2 it is expected that its skin tolerability will be further improved compared to Example 1.

Example 8:

The relatively high drug concentration mediated by the invention's technology might be able to induce therapeutic effects unrelated to the well known prostaglandin-mediated pharmacology. Those effects would be related to direct effects to the nociceptors.

Histamine is often used in the art to induce a neurogenic flare reaction. Recent evidence suggests that there is an itch-specific neural pathway. Human histamine-sensitive C-fibers (small unmyelinated primary afferents) have been characterised by mechanical insensitivity, slow conduction velocity, and huge receptive fields [Schmelz et al., 1997].

The composition of Example 1 was used to study the effectiveness of inventive preparation in reducing histamine-induced itch. This test was part of the study described in Example 4.

The study involved 38 healthy volunteers, who received either an itch-inducing dose of histamine or placebo. Treatment with the formulation of Example 1 showed a trend towards reducing the itching caused by the histamine, as shown by the AUC for Example 1, least square mean: 45.15 (95 % ci: 42.46 – 47.83) compared to placebo, least square mean: 47.83 (95 % ci: 45.15-50.52).

Example 9

The depigmentation effect of the invention was seen in the context of a clinical trial.

- A 47 year old women with naturally pigmented, brown skin, used a 2.29 %
 5 ketoprofen gel based on Transfersomes ®, as described in US patent application
 serial-no. 10/357 617. More specifically, the formulation was closely based on
 Example 32 of said US patent application, comprising

	Weight-%	
	2.290	Ketoprofen
10	6.870	Soy Phosphatidylcholine (SPC)
	0.850	Polysorbate (Tween 80)
	3.651	Ethanol 96 %
	0.930	NaOH (sodium hydroxide)
	0.235	Phosphate buffer salts
15	0.50	Sodium metabisulphite
	0.20	Butylhydroxytoluene (BHT)
	0.100	Disodium edentate (EDTA)
	0.250	Methyl parahydroxybenzoate
	0.525	Benzyl alcohol
20	0.100	Linalool
	1.250	Carbomer (Carbopol 980)
	3.00	Glycerol
	79.879	Water

- 25 The test person was affected by epicondylitis of the right hand, and received
 concomitant corresponding medication that was unchanged during the time of
 treatment with the gel. The ketoprofen transfersome-® gel was repeatedly used over
 a period of nine days.

- 35 -

Over this time period, a profound depigmentation of the skin topically treated with the ketoprofen gel, became visible. In the skin areas where the gel was applied, the pigmentation was largely destroyed, so that the skin took a white or "bleached" appearance.

5

After nine days, the use of the transfersome gel was discontinued. The depigmentation effect persisted for more than two months thereafter.

10 It is assumed that the usefulness of the invention is not limited to ketoprofen, and extends at least to the NSAIDs' class of pharmaceutically active substances. It may be expected that besides ketoprofen, those NSAIDs would be useful in the context of the present invention which show similar depigmentation effectiveness on damaged skin.

15 It is further expected that beyond NSAIDs, the invention can be used with other drugs that are known to cause depigmentation or hypopigmentation on damaged skin. It is generally assumed that the invention can be practised with any type of active, in a suitable concentration, that may cause depigmentation, especially by inducing melanocyte apoptosis.

20

It is also expected that the invention can be used to stimulate pigmentation, where this is desired. This would likely require the application of suitable (low) doses of corresponding actives known to stimulate pigment production by the melanocytes.

25 The present invention therefore has important potential usefulness in cosmetic as well as medical applications, including the treatment of skin cancer.

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Clinical details of the intended treatment will vary, depending on the desired effect, and still need to be studied. Presently, the available evidence is a case report, as described below. Based on general experience and skill, it is however expected that the presently available observations can be extended other patients, and are not

5 limited to any specific patient group.

C L A I M S

- 5 1. The use of extended surface aggregates (ESAs) comprising
at least one first amphipathic component, which is a basic aggregate-forming
component, and at least one second amphipathic component, which decreases
aggregate sensitivity to physical stress, including stress created by enforced passage
of said ESAs through pores with an average pore diameter at least 50 % smaller than
10 the average diameter of the ESAs before said passage,
such that the average ESA diameter change induced by such physical stress is
reduced by 10 % or more, compared to the diameter change induced by such stress in
a reference system comprising just the first or just the second aggregate component,
in the manufacture of a pharmaceutical preparation for enduring treatment of
15 pathological mammalian skin conditions, including skin irritation, skin inflammation
and/or skin damage after topical application, for modifying skin pigmentation and/or
for treatment of skin itch.
2. The use of any preceding claim, wherein the at least one second
20 amphipathic component is an NSAID, such as ketoprofen, ibuprofen, diclofenac,
indomethacin, naproxen or piroxicam.
3. The use of any preceding claim, wherein the first amphipathic
component is selected from the group consisting of phospholipids, such as
25 phosphatidylcholines, phosphatidylethanolamines, phosphatidylglycerols,

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phosphatidylinositols, phosphatidic acids, phosphatidylserines, sphingomyelins, shingophospholipids, glycosphingolipids, cerebroside, ceramidpolyhexosides, suphatides, sphingoplasmalogenes, or gangliosides.

5 4. The use of any preceding claim, wherein the at least one second amphipathic component is selected from the group of non-ionic surfactants, and preferably is a polyethyleneglycol-sorbitan-long fatty chain ester, a polyethyleneglycol-long fatty chain ester or -ether, a polyhydroxyethylen-long fatty chain ester or -ether, or a surfactant-like non-ionic phospholipid.

10

 5. The use of any preceding claim, wherein the ESA comprises at least one first amphipathic component, which is an aggregate-forming component; a second amphipathic component which is an aggregate-deformability increasing component, and a third amphipathic component, which is an aggregate-deformability
15 increasing component and chemically different from said second component.

 6. The use of any preceding claim, wherein said first amphipathic component is capable of forming bilayer membranes, and said second and third amphipathic components are chosen to exert a membrane-destabilising effect on said
20 bilayer membranes, which decreases the sensitivity to stress of said membranes when passing through said pores.

 7. The use of claim 6, wherein said components are chosen to provide a synergistic effect of said combined second and third component in decreasing said
25 membrane sensitivity to stress.

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8. The use of any preceding claim, wherein the first amphipathic component is a phosphatidylcholine and the second or third amphipathic component is an NSAID, such as ketoprofen, diclofenac, ibuprofen, indomethacin, naproxen, or piroxicam.

5

9. The use of any preceding claim, wherein the average ESA diameter before the ESAs penetrate the pores, is at least 40 % larger than the average pore diameter.

10

10. The use of any preceding claim, wherein the change in the average ESA diameter after ESA exposure to said physical stress is at least 20 % smaller than the change measured with the reference system which lacks one, two, or more of said ESA components.

15

11. The use of any preceding claim, wherein the first component and the second component differ in solubility in the liquid medium at least 10-fold, on average.

20

12. The use of any preceding claim, wherein the second component and the third component differ in solubility, on average, at least 2-fold.

13. The use of any preceding claim, wherein the total dry mass of the amphipathic components is between 0.01 weight-% and 50 weight-%.

- 40 -

14. The use according to any preceding claim, wherein the extended surfaces formed by the amphipathic components have an average curvature corresponding to an average diameter between 15 nm and 5000 nm.

5 15. The use according to any previous claim, said ESAs comprising a lower aliphatic alcohol, preferably n-propanol, isopropanol, 2-propanol, n-butanol, 2-butanol, 1,2-propanediol, 1,2-butanediol, or ethanol as a further aggregate or membrane destabilising component.

10 16. The use of any preceding claim, wherein the composition comprises an NSAID and the bulk pH value of the preparation is above the logarithm of the apparent dissociation constant (pKa) of the NSAID drug in solution and in extended surface aggregates, and the latter pKa is higher than the former.

15 17. The use of claim 16, wherein the bulk pH value is between 6.4 and 8.3, more preferably between 6.7 and 8 and most preferably between 7 and 7.7.

18. The use of any preceding claim, wherein the bulk ionic strength of the preparation is between 0.005 and 0.3, preferably between 0.01 and 0.2 and most
20 preferably between 0.05 and 0.15.

19. The use of any preceding claim, wherein the formulation viscosity is between 50 mPa s and 30.000 mPa s, preferably between 100 mPa s and 10.000 mPa s, more preferably between 200 mPa s.

25

20. The use of any preceding claim, wherein a membrane-forming phospholipid first component and a membrane-destabilising NSAID second component are present in the suspension in a relative molar ratio between 10/1 and 1/2.

5

21. The use of any preceding claim, wherein a membrane forming phospholipid first component and a membrane adaptability increasing surfactant second component are present in the suspension in a relative molar ratio between 40/1 and 1/4.

10

22. The use of any preceding claim, said preparation comprising an NSAID active selected from ketoprofen, diclofenac, flurbiprofen, piroxicam and other actives of similar potency, at an applied drug dose per unit area of mammalian skin, between $0.0001 \text{ mg cm}^{-2}$ and 1 mg cm^{-2} , preferably between $0.0005 \text{ mg cm}^{-2}$ and 0.5 mg cm^{-2} , more preferably between 0.001 mg cm^{-2} and 0.3 mg cm^{-2} and most preferably between 0.005 mg cm^{-2} and 0.1 mg cm^{-2} .

15

23. The use of claim 22, at an applied (total) unit dose between 1 mg and 100 mg, preferably between 5 mg and 50 mg and most preferably between 5 mg and 30 mg drug substance.

20

24. The use of any one of claims 1 to 23, said preparation comprising an NSAID active selected from diclofenac, ketoprofen, naproxen, indomethacin, ibuprofen, and other actives of similar potency, at an applied drug substance dose per unit area of mammalian skin between 0.05 mg/cm^2 and 10 mg/cm^2 , preferably

25

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between 0.1 mg/cm² and 5 mg/cm², more preferably between 0.2 mg/cm² and 3 mg/cm² and most preferably between 0.2 mg/cm² and 0.6 mg/cm².

25. The use of any one of claims 1 to 23, said preparation comprising an
5 active selected from salicylates, pyrazolene derivatives such as pherylbutazone, tolmetine and other actives of similar potency, at an applied drug substance dose per unit area of mammalian skin between 0.5 mg/cm² and 50 mg/cm², preferably between 1 mg/cm² and 30 mg/cm² and most preferably between 2 mg/cm² and 6 mg/cm².

10

26. The use of any preceding claim, said pathological skin conditions including skin irritation, pain, itching, inflammation and/or skin damage.

27. The use of any preceding claim, said skin pigment modification
15 including cosmetic skin depigmentation, treatment of skin hyperpigmentation, or treatment of undesired pigment cell proliferation.

28. The use of any preceding claim, said pharmaceutical preparation being formulated for topical application, e.g. as a non-occlusive patch.

20

29. A method for treating peripheral pain and/or inflammation by applying a pharmaceutical preparation as defined in any preceding claim on the skin of a warm blooded mammal.

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30. The method according to claim 29, wherein the pharmaceutical preparation is applied in a non-occlusive patch.

31. A kit comprising, in a tube, a spray can or a roller-ball container, a patch or some other packaged form, at least one unit dose of the pharmaceutical preparation as defined in any one of claims 1 to 28.

32. The use or method according to any one of claims 1 to 39, wherein relative ratios of said first, said second, and said third component in the topically used pharmaceutical preparation are selected so as to control the duration of active ingredient presence in the target skin tissue and the outcome of skin treatment.

33. The use, method or unit dose according to any one of claims 1 to 32, wherein a suspension of drug free ESAs is loaded with an NSAID to be associated therewith during the day prior to an administration, preferably 360 min, more preferably 60 min, even more preferably 30 min and most preferably 5 min before administering the resulting formulation on the skin.

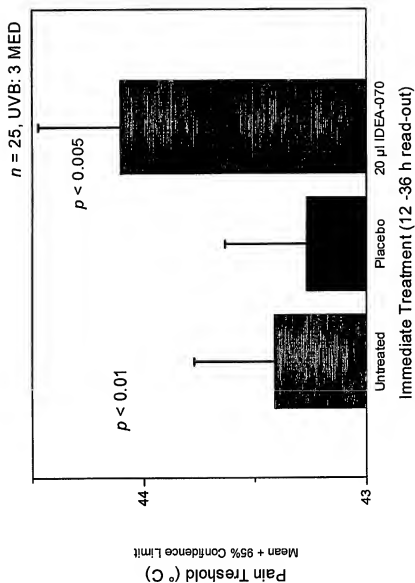


Fig. 1

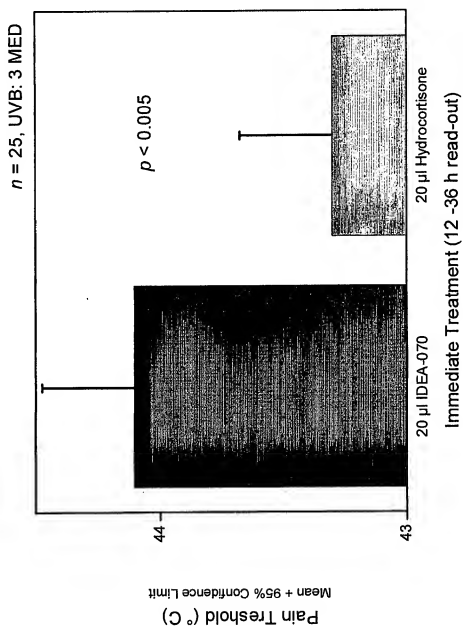


Fig. 2

3 / 10

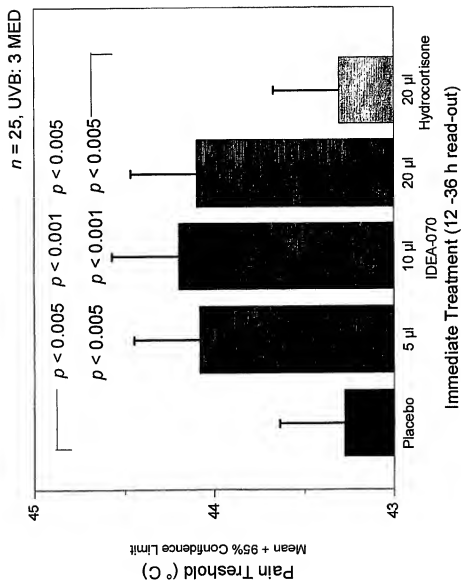


Fig. 3

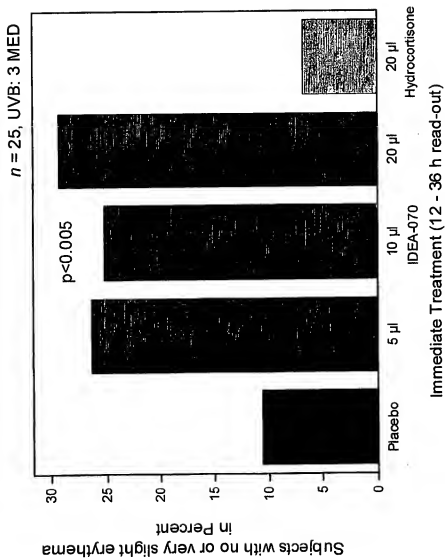


Fig. 4

5 / 10

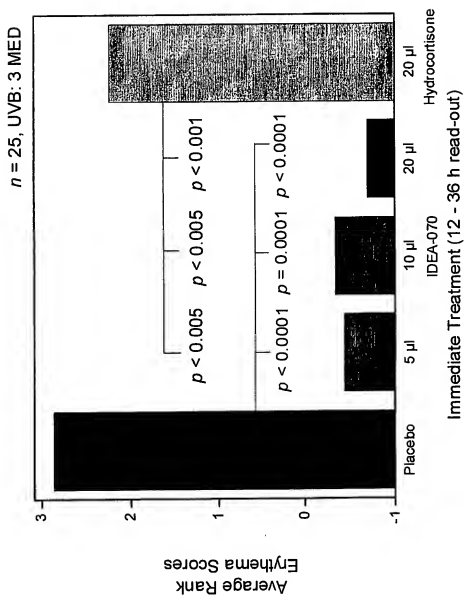


Fig. 5

6 / 10

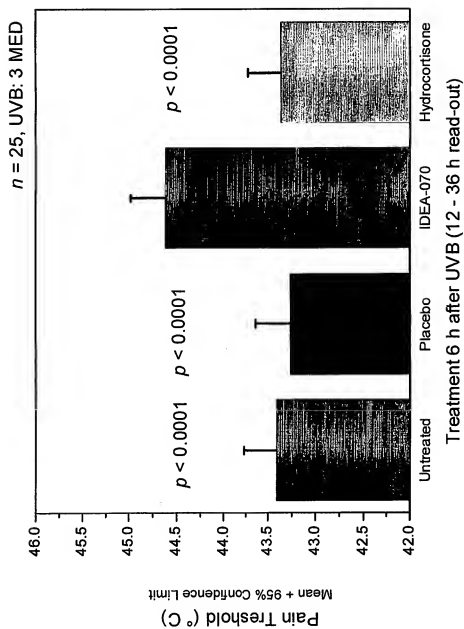
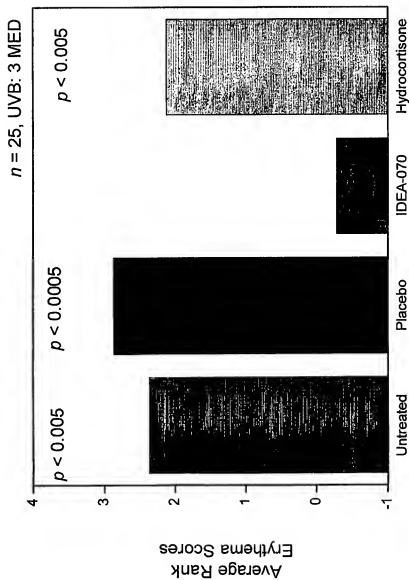


Fig. 6

7 / 10



Treatment 6 h after UVB (12 - 36 h read-out)

Fig. 7

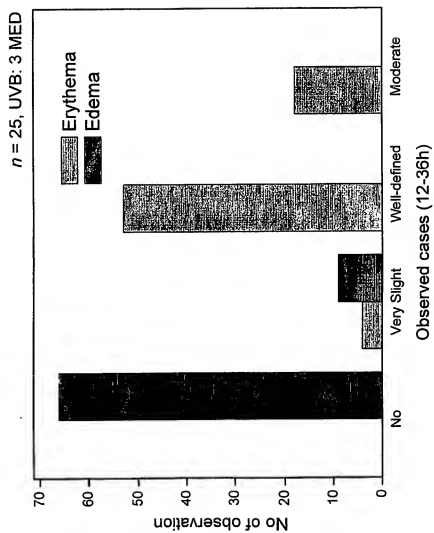


Fig. 8

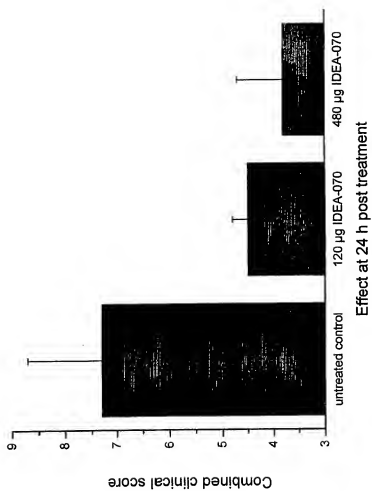


Fig. 9

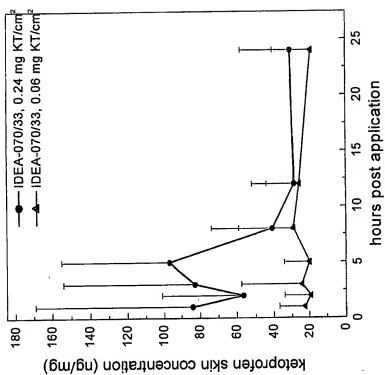


Fig. 10

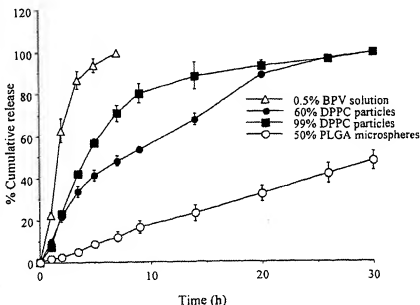
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- (71) Applicant: **MASSACHUSETTS INSTITUTE OF TECHNOLOGY** [US/US]; 77 Massachusetts Avenue, Cambridge, MA 02139 (US).
- (72) Inventors: **KOHANE, Daniel, S.**; 41 Leslie Road, Newton, MA 02466 (US). **LIPP, Michael, M.**; 1015 S. Artery
- (74) Agent: **BAKER, C., Hunter**; Choate, Hall & Stewart, Exchange Place, 53 State Street, Boston, MA 02109 (US).
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(54) Title: LIPID-PROTEIN-SUGAR PARTICLES FOR DRUG DELIVERY



(57) Abstract: Lipid-protein-sugar particles (LPSPs) are provided as a vehicle for drug delivery. Any therapeutic, diagnostic, or prophylactic agent may be encapsulated in a lipid-protein-sugar matrix to form microparticles. Preferably the diameter of the LPSP ranges from 50 nm to 10 micrometers. The particles may be prepared using any known lipid (e.g., DPPC), protein (e.g., albumin), or sugar (e.g., lactose). Methods of preparing and administering the particles are also provided. Methods of providing a nerve block are also provided by administering LPSPs with a local anesthetic (e.g., bupivacaine) within the vicinity of a nerve.

LIPID-PROTEIN-SUGAR PARTICLES FOR DRUG DELIVERY

Related Applications

5 The present application claims priority to co-pending provisional application, USN 60/240,636, filed October 16, 2000, which is incorporated herein by reference.

Government Support

10 The work described herein was supported, in part, by grants from the National Institutes of Health (GM00684-01). The United States government may have certain rights in the invention.

Background of the Invention

15 The delivery of a drug to a patient with controlled-release of the active ingredient has been an active area of research for decades and has been fueled by the many recent developments in polymer science and the need to deliver more labile pharmaceutical agents such as nucleic acids, proteins, and peptides. Biodegradable particles have been developed as sustained release vehicles used in the administration of small molecule drugs as well as protein and peptide drugs and nucleic acids

20 (Langer *Science* 249:1527-1533, 1990; Mulligan *Science* 260:926-932, 1993; Eldridge *Mol. Immunol.* 28:287-294, 1991; each of which is incorporated herein by reference). The drugs are typically encapsulated in a polymer matrix which is biodegradable and biocompatible. As the polymer is degraded and/or as the drug diffuses out of the polymer, the drug is released into the body. Typical polymers used

25 in preparing these particles are polyesters such as poly(glycolide-co-lactide) (PLGA),

polyglycolic acid, poly- β -hydroxybutyrate, and polyacrylic acid ester. These particles have the additional advantage of protecting the drug from degradation by the body. These particles depending on their size, composition, and the drug being delivered can be administered to an individual using any route available.

5 Biocompatibility is of special importance when a sustained release vehicle such as microparticles is used for local delivery of a drug to sensitive or vital structures (*e.g.*, nerves, eyes, atria, brain, uterus), particularly if the dwell time of the polymeric device in the target tissue is much longer than the clinical efficacy of the delivered drug. In the case of local anesthesia, the problem is further exacerbated by
10 the large loads of drug and polymer that typically must be delivered in order to achieve effective and prolonged nerve blockade given the relatively low potency of most conventional local anesthetics.

While local anesthetics are often intended to last for the relatively brief duration of a dental or surgical procedure (*e.g.*, a few hours), there are many instances
15 where a more prolonged blockade is desirable. Controlled release technology can prolong the effect of the drug and improve the therapeutic index, and therefore lends itself naturally to the problem of providing prolonged duration local anesthesia. A large number of approaches have been tried (for example, see Boedecker *et al.* "Ultra-long-duration local anesthesia produced by injection of lecithin-coated tetracaine microcrystals" *J. Clin. Pharmacol.* 34:699-702, 1994; Curley *et al.* "Prolonged
20 regional nerve blockade. Injectable biodegradable bupivacaine/polyester microspheres" *Anesthesiology* 84:1401-1410, 1996; Grant *et al.* "Prolonged analgesia with liposomal bupivacaine in a mouse model" *Reg. Anesth.* 19:264-269, 1994; Kirkpatrick *et al.* "Long duration local anesthesia with lecithin-coated microdroplets

of methoxyflurane: Studies with rat skin" *Reg. Anesth.* 16:164-172, 1991; each of which is incorporated herein by reference).

What is needed is a drug delivery vehicle that will provide prolonged delivery of an agent and will not lead to inflammatory reactions and is biocompatible with the
5 tissue to which the agent is being delivered.

Summary of the Invention

The present invention provides a system for delivering an agent encapsulated in a lipid-protein-sugar matrix to an individual. The encapsulated agent may be a
10 diagnostic, prophylactic, or therapeutic agent. In a preferred embodiment, the agent is encapsulated in the lipid-protein-sugar matrix to form small particles for administration to the individual. Typically the size of these particles ranges from 5 micrometers to 50 nanometers. The lipid-protein-sugar particles (LPSP) typically comprise a surfactant or phospholipid or similar hydrophic or amphiphilic molecule; a
15 protein; a simple and/or complex sugar; and the agent to be delivered. In a particularly preferred embodiment, the lipid is dipalmitoylphosphatidylcholine (DPPC), the protein is albumin, and the sugar is lactose. In another particularly preferred embodiment, a synthetic polymer is substituted for at least one of the components of the LPSPs—lipid, protein, and/or sugar. One advantage of LPSPs
20 over other polymeric vehicles is that the compounds used to create LPSPs are naturally occurring and therefore have improved biocompatibility compared to other polymers such as PLGA. The LPSPs may be prepared using techniques known in the art including spray drying.

In another aspect, the present invention provides a method of administering the inventive LPSPs and pharmaceutical compositions comprising LPSPs to an individual human or animal. The LPSPs once prepared can be administered to the individual by any means known in the art including, for example, intravenous
5 injection, intradermal injection, rectally, orally, intravaginally, inhalationally, *etc.* Preferably, administration of the encapsulated agent provides sustained release of the agent.

In yet another aspect, the present invention provides a method of administering a nerve block. The agent to be delivered may be an anesthetic such as
10 an amine-amide-containing anesthetic (*e.g.*, bupivacaine, lidocaine). LPSPs containing these agents may be delivered in the vicinity of a nerve to provide local anesthesia of a desired area.

15 Definitions

"Adjuvant": The term adjuvant refers to any compound which is a nonspecific modulator of the immune response. In certain preferred embodiments, the adjuvant stimulates the immune response. Any adjuvant may be used in accordance with the present invention. A large number of adjuvant compounds is known; a useful
20 compendium of many such compounds is prepared by the National Institutes of Health and can be found on the world wide web (<http://www.niaid.nih.gov/daids/vaccine/pdf/compendium.pdf>, incorporated herein by reference; see also Allison *Dev. Biol. Stand.* 92:3-11, 1998; Unkeless *et al. Annu. Rev.*

Immunol. 6:251-281, 1998; and Phillips *et al. Vaccine* 10:151-158, 1992, each of which is incorporated herein by reference).

“Animal”: The term animal, as used herein, refers to humans as well as non-human animals, including, for example, mammals, birds, reptiles, amphibians, and fish. Preferably, the non-human animal is a mammal (*e.g.*, a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a primate, or a pig). An animal may be a transgenic animal.

“Associated with”: When two entities are “associated with” one another as described herein, they are linked by a direct or indirect covalent or non-covalent interaction. Preferably, the association is covalent. Desirable non-covalent interactions include hydrogen bonding, van der Waals interactions, hydrophobic interactions, magnetic interactions, electrostatic interactions, *etc.*

“Biocompatible”: The term “biocompatible”, as used herein is intended to describe compounds that are not toxic to cells. Compounds are “biocompatible” if their addition to cells *in vitro* results in less than or equal to 20 % cell death and do not induce inflammation or other such adverse effects *in vivo*.

“Biodegradable”: As used herein, “biodegradable” compounds are those that, when introduced into cells, are broken down by the cellular machinery into components that the cells can either reuse or dispose of without significant toxic effect on the cells (*i.e.*, fewer than about 20 % of the cells are killed).

“Effective amount”: In general, the “effective amount” of an active agent or LPSs refers to the amount necessary to elicit the desired biological response. As will be appreciated by those of ordinary skill in this art, the effective amount of LPSs may vary depending on such factors as the desired biological endpoint, the

agent to be delivered, the composition of the encapsulating matrix, the target tissue, *etc.* For example, the effective amount of LPSPs containing a local anesthetic to be delivered to provide a nerve block is the amount that results in a reduction in sensation of a desired area for a desired length of time. In another example, the effective amount of LPSPs containing an antigen to be delivered to immunize an individual is the amount that results in an immune response sufficient to prevent infection with an organism having the administered antigen.

“Peptide” or “protein”: According to the present invention, a “peptide” or “protein” comprises a string of at least three amino acids linked together by peptide bonds. The terms “protein” and “peptide” may be used interchangeably. Peptide may refer to an individual peptide or a collection of peptides. Inventive peptides preferably contain only natural amino acids, although non-natural amino acids (*i.e.*, compounds that do not occur in nature but that can be incorporated into a polypeptide chain; see, for example, <http://www.cco.caltech.edu/~dadgrp/Unnatstruct.gif>, which displays structures of non-natural amino acids that have been successfully incorporated into functional ion channels) and/or amino acid analogs as are known in the art may alternatively be employed. Also, one or more of the amino acids in an inventive peptide may be modified, for example, by the addition of a chemical entity such as a carbohydrate group, a phosphate group, a farnesyl group, an isofarnesyl group, a fatty acid group, a linker for conjugation, functionalization, or other modification, *etc.* In a preferred embodiment, the modifications of the peptide lead to a more stable peptide (*e.g.*, greater half-life *in vivo*). These modifications may include cyclization of the peptide, the incorporation of D-amino acids, *etc.* None of

the modifications should substantially interfere with the desired biological activity of the peptide.

“Polynucleotide” or “oligonucleotide”: Polynucleotide or oligonucleotide refers to a polymer of nucleotides. Typically, a polynucleotide comprises at least
5 three nucleotides. The polymer may include natural nucleosides (*i.e.*, adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine), nucleoside analogs (*e.g.*, 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, 5-methylcytidine, C-5 propynyl-cytidine, C-5 propynyl-uridine, 2-aminoadenosine, C5-bromouridine,
10 C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, and 2-thiocytidine), chemically modified bases, biologically modified bases (*e.g.*, methylated bases), intercalated bases, modified sugars (*e.g.*, 2'-fluororibose, ribose, 2'-deoxyribose,
15 arabinose, and hexose), or modified phosphate groups (*e.g.*, phosphorothioates and 5'-N-phosphoramidite linkages).

“Small molecule”: As used herein, the term “small molecule” refers to organic compounds, whether naturally-occurring or artificially created (*e.g.*, via chemical synthesis) that have relatively low molecular weight and that are not
20 proteins, polypeptides, or nucleic acids. Typically, small molecules have a molecular weight of less than about 1500 g/mol. Also, small molecules typically have multiple carbon-carbon bonds. Known naturally-occurring small molecules include, but are not limited to, penicillin, erythromycin, taxol, cyclosporin, and rapamycin. Known

synthetic small molecules include, but are not limited to, ampicillin, methicillin, sulfamethoxazole, and sulfonamides.

“Sugar”: The term “sugar” refers to any carbohydrate. Sugars useful in the present invention may be simple or complex sugars. Sugars may be monosaccharides (e.g., dextrose, fructose, inositol), disaccharides (e.g., sucrose, saccharose, maltose, lactose), or polysaccharides (e.g., cellulose, glycogen, starch). Sugars may be obtained from natural sources or may be prepared synthetically in the laboratory. In a preferred embodiment, sugars are aldehyde- or ketone-containing organic compounds with multiple hydroxyl groups.

“Surfactant”: Surfactant refers to any agent which preferentially absorbs to an interface between two immiscible phases, such as the interface between water and an organic solvent, a water/air interface, or an organic solvent/air interface. Surfactants usually possess a hydrophilic moiety and a hydrophobic moiety, such that, upon absorbing to microparticles, they tend to present moieties to the external environment that do not attract similarly-coated particles, thus reducing particle agglomeration. Surfactants may also promote absorption of a therapeutic or diagnostic agent and increase bioavailability of the agent. The term surfactant may be used interchangeably with the terms lipid and emulsifier in the present application.

20

Brief Description of the Drawing

Figure 1 is a scanning electron micrograph of spray-dried lipid-protein particles (60% dipalmitoylphosphatidylcholine) as prepared.

Figure 2 shows the cumulative release from a dialysis tube of bupivacaine encapsulated in 10% (w/w) bupivacaine lipid-protein particles with 60% (●) or 99% (■) of the excipients being dipalmitoylphosphatidylcholine, or an equivalent amount of 0.5% (w/v) bupivacaine in solution (Δ). Also shown is release from 50% (w/w) bupivacaine PLGA microsphere (O). Data shown are means with standard deviations. n=4 for all points.

Figure 3 shows the comparison of the durations of sensory and motor blockade for 10% (w/w) bupivacaine lipid-protein (●), 50% (w/w) bupivacaine PLGA microspheres (O), and 0.5% (w/v) bupivacaine in solution (Δ). Points falling above the diagonal line bisecting the graph represent a relative sensory predominance in nerve blockade, while those falling below have motor predominance.

Figure 4 shows the time course of thermal latency in the uninjected leg following sciatic nerve block in animals injected with 10% (w/w) bupivacaine lipid-protein particles (●) and in animals injected with 50% (w/w) bupivacaine PLGA microspheres (O). Here thermal latency in the uninjected (contralateral) leg is used as a measure of systemic drug distribution. Data shown are means with standard deviations. None of the differences in latency between the two groups were statistically significant.

Figure 5 shows a photomicrograph of a control nerve at low power (A). Note the absence of inflammatory cells outside the neural sheath (perineurium). Figure 5B shows a high power view (400X) of connective tissue outside the perineurium. Note the looseness and low cellularity of the connective tissue.

Figure 6 shows PLGA microspheres at the sciatic nerve. The microspheres are seen in close proximity to the nerve, but outside the nerve sheath. N = sciatic nerve. BV = blood vessel. M = muscle. MS = microspheres.

Figure 7 shows a summary of histological findings over time. Figure 7A shows dissection scores. Median +/- 25th and 75th percentiles. No statistically significant difference between the groups was observed. Figure 7B shows the prevalence of particle residue. Figure 7C shows areas of inflammation at two weeks. Median +/- 25th and 75th percentiles. In the PLGA group, the area was too large and amorphous at 4 days to be dissected *en bloc*. At 7 months there was no obvious entity to measure in either group. Figure 7D shows the cell density of the inflammatory response. Data shown are means with standard deviations. In summary, there were robust differences at two weeks post injection.

Figure 8 shows photomicrographs four days after particle injection. Figure 8A shows the injection of PLGA microspheres. The microspheres are surrounded by a dense infiltrate, surrounded by intense granulomatous inflammation, with some foreign body giant cells. In general (and at all time points examined), the inflammatory response to PLGA microspheres was confined to clearly demarcated pockets that were firm and slightly gritty to palpation. Figure 8B shows injection of LPSs. A dense inflammatory reaction that was more diffuse than that resulting from the injection of PLGA microspheres was observed. Pockets of particle were smaller and softer to the touch. In both cases, inflammation also involved adjacent muscle and epineural adipose tissue. The injection site had necrotic muscle fibers, myophagocytosis, and myocyte regeneration--all signs of acute muscle injury.

Figure 9 shows photomicrographs two weeks after particle injection. Figure 9A shows the injection of PLGA microspheres. The histological appearance was very similar to that at 4 days (and at 8 weeks), with lymphocytes, macrophages, and giant cells in granulomas. The asterisk denotes a "ghost" of a microsphere. One sample in this group had active myositis. Figure 9B shows the injection of LPSPs. There was a small loose, predominantly lymphocytic infiltrate. In both cases, histological appearance was the same for particles without drug.

Figure 10 shows ectopic particles. Figure 10A shows sub-perineurial microspheres. Arrows indicate "ghosts" of microspheres. Figure 10B shows a pocket of microspheres approximately 0.4 mm x 4 mm x 8 mm found at knee of rat (about 2.5 cm from site of injection).

Figure 11 shows the release of the anticonvulsant muscimol from LPSPs loaded with the drug. An *in vitro* dialysis assay was used to determine the release of the drug from the particles in comparison to free muscimol.

Figure 12 shows the release of the vasodilator, nifedipine, from LPSPs using an *in vitro* assay simulating physiological conditions.

Detailed Description of Certain Preferred Embodiments of the Invention

The present invention provides a system including a pharmaceutical composition of lipid-protein-sugar particles (LPSP) containing an agent as well as methods of preparing and administering the LPSPs. Agents administered using LPSPs preferably have a sustained release profile and may be administered to any animal to be treated, diagnosed, or prophylaxed. The matrix of the inventive LPSPs

also are preferably substantially biocompatible and preferably cause minimal inflammatory reaction, and the degradation products are preferably easily eliminated by the body (*i.e.*, the components of the LPSPs matrix are biodegradable).

5 *Agent*

The agents to be delivered by the system of the present invention may be therapeutic, diagnostic, or prophylactic agents. Any chemical compound to be administered to an individual may be delivered using LPSPs. The agent may be a small molecule, organometallic compound, nucleic acid, protein, peptide, metal, an
10 isotopically labeled chemical compound, drug, vaccine, immunological agent, *etc.*

In a preferred embodiment, the agents are organic compounds with pharmaceutical activity. In another embodiment of the invention, the agent is a clinically used drug. In a particularly preferred embodiment, the drug is an antibiotic, anti-viral agent, anesthetic, steroidal agent, anti-inflammatory agent, anti-neoplastic
15 agent, antigen, vaccine, antibody, decongestant, antihypertensive, sedative, birth control agent, progestational agent, anti-cholinergic, analgesic, anti-depressant, anti-psychotic, β -adrenergic blocking agent, diuretic, cardiovascular active agent, vasoactive agent, non-steroidal anti-inflammatory agent, nutritional agent, *etc.* In a particularly preferred embodiment, the agent is a local anesthetic. Particularly
20 preferred anesthetics are amine-amide containing anesthetics. Anesthetics include, but are not limited to, lidocaine, procaine, dibucaine, tetracaine, bupivacaine, mepivacaine, benzocaine, etidocaine, prilocaine, ropivacaine, proparacaine, pramoxine, chloroprocaine, cocaine, and articaine.

The agents delivered may be a mixture of pharmaceutically active agents. For example, a local anesthetic may be delivered in combination with an anti-inflammatory agent such as a steroid. Local anesthetics may also be administered with vasoactive agents such as epinephrine. To give but another example, an antibiotic may be
5 combined with an inhibitor of the enzyme commonly produced by bacteria to inactivate the antibiotic (e.g., penicillin and clavulanic acid).

Diagnostic agents include gases; commercially available imaging agents used in positron emissions tomography (PET), computer assisted tomography (CAT), single photon emission computerized tomography, x-ray, fluoroscopy, and magnetic
10 resonance imaging (MRI); and contrast agents. Examples of suitable materials for use as contrast agents in MRI include gadolinium chelates, as well as iron, magnesium, manganese, copper, and chromium. Examples of materials useful for CAT and x-ray imaging include iodine-based materials.

Prophylactic agents include vaccines. Vaccines may comprise isolated
15 proteins or peptides, inactivated organisms and viruses, dead organisms and virus, genetically altered organisms or viruses, and cell extracts. Prophylactic agents may be combined with interleukins, interferon, cytokines, and adjuvants such as cholera toxin, alum, Freund's adjuvant, etc. Prophylactic agents include antigens of such bacterial organisms as *Streptococcus pneumoniae*, *Haemophilus influenzae*,
20 *Staphylococcus aureus*, *Streptococcus pyogenes*, *Corynebacterium diphtheriae*, *Listeria monocytogenes*, *Bacillus anthracis*, *Clostridium tetani*, *Clostridium botulinum*, *Clostridium perfringens*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Streptococcus mutans*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Haemophilus parainfluenzae*, *Bordetella pertussis*, *Francisella tularensis*, *Yersinia pestis*, *Vibrio*

- cholerae*, *Legionella pneumophila*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Treponema pallidum*, *Leptospirosis interrogans*, *Borrelia burgdorferi*, *Camphylobacter jejuni*, and the like; antigens of such viruses as smallpox, influenza A and B, respiratory syncytial virus, parainfluenza, measles, HIV, varicella-zoster, herpes simplex 1 and 2, cytomegalovirus, Epstein-Barr virus, rotavirus, rhinovirus, adenovirus, papillomavirus, poliovirus, mumps, rabies, rubella, coxsackieviruses, equine encephalitis, Japanese encephalitis, yellow fever, Rift Valley fever, hepatitis A, B, C, D, and E virus, and the like; antigens of fungal, protozoan, and parasitic organisms such as *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Candida albicans*, *Candida tropicalis*, *Nocardia asteroides*, *Rickettsia rickettsii*, *Rickettsia typhi*, *Mycoplasma pneumoniae*, *Chlamydia psittaci*, *Chlamydia trachomatis*, *Plasmodium falciparum*, *Trypanosoma brucei*, *Entamoeba histolytica*, *Toxoplasma gondii*, *Trichomonas vaginalis*, *Schistosoma mansoni*, and the like. These antigens may be in the form of whole killed organisms, peptides, proteins, glycoproteins, carbohydrates, or combinations thereof.

Microparticle Excipients

- The agent is preferably encapsulated in a matrix comprising lipid, protein, and sugar to form microparticles. In a preferred embodiment, the diameter of the microparticles is less than 10 micrometers and more preferably less than 5 micrometers. The size of the microparticles and distribution of sizes may be selected by one of ordinary skill in the art based on the agent being delivered, the target tissue, route of administration, method of uptake by the cells, etc. In certain embodiments, one of the three components may be eliminated from the matrix. In other

embodiments, a synthetic polymer (*e.g.*, poly(lactic-co-glycolic acid) (PLGA), polyglycolic acid (PGA), polesters, polyanhydrides, polyamides, *etc.*) is used as a substitute for at least one of the components of the LPSPs. The specific ratios of the excipients may range widely depending on factors including size of particle, porosity of particle, agent to be delivered, desired agent release profile, target tissue, *etc.* One of ordinary skill in the art may test a variety of ratios and specific components to determine the composition correct for the desired purpose. Any known lipid, protein, and sugar, natural or unnatural, may be used to prepare the inventive microparticles.

10 *Lipids or Surfactants or Emulsifiers*

The lipid portion of the matrix of the inventive LPSPs is thought to bind the particle together. The hydrophobicity of the lipid may also contribute to the slow release of the encapsulated drug. The percent of lipid in the matrix (excluding the agent) may range from 0% to 99%, more preferably from 3% to 99%.

15 Any lipid, surfactant, or emulsifier known in the art is suitable for use in making the inventive microparticles. Such surfactants include, but are not limited to, phosphoglycerides; phosphatidylcholines; dipalmitoyl phosphatidylcholine (DPPC); dioleoylphosphatidyl ethanolamine (DOPE); dioleoyloxypropyltriethylammonium (DOTMA); dioleoylphosphatidylcholine; cholesterol; cholesterol ester;

20 diacylglycerol; diacylglycerolsuccinate; diphosphatidyl glycerol (DPPG); hexanadecanol; fatty alcohols such as polyethylene glycol (PEG); polyoxyethylene-9-lauryl ether; a surface active fatty acid, such as palmitic acid or oleic acid; fatty acids; fatty acid amides; sorbitan trioleate (Span 85) glycocholate; surfactin; a poloxomer; a sorbitan fatty acid ester such as sorbitan trioleate; lecithin; lysolecithin;

phosphatidylserine; phosphatidylinositol; sphingomyelin; phosphatidylethanolamine (cephalin); cardiolipin; phosphatidic acid; cerebrosides; dicetylphosphate; dipalmitoylphosphatidylglycerol; stearylamine; dodecylamine; hexadecyl-amine; acetyl palmitate; glycerol ricinoleate; hexadecyl stearate; isopropyl myristate;

5 tyloxapol; poly(ethylene glycol)5000-phosphatidylethanolamine; and phospholipids.

The lipid component may also be a mixture of different lipid molecules. These lipid may be extracted and purified from a natural source or may be prepared synthetically in a laboratory. In a preferred embodiment, the lipids are commercially available.

10 *Protein*

The protein component of the encapsulating matrix may be any protein or peptide. The protein of the LPSPs presumably plays a structural role in the microparticles. Proteins useful in the inventive system include albumin, gelatin, whole cell extracts, antibodies, and enzymes (*e.g.*, glucose oxidase, *etc.*). The protein

15 may be chosen based on known interactions between the protein and the agent being delivered. For example, bupivacaine is known to bind to albumin in the blood; therefore, albumin would be a logical choice in choosing a protein from which to prepare microparticles containing bupivacaine. The percentage of protein in the matrix (excluding the agent to be delivered) may range from 0% to 99%, more

20 preferably 1% to 80%, and most preferably from 1% to 60%.

In certain preferred embodiments, the agent to be delivered is a protein. In these embodiments, the protein to be delivered may make up all or a portion of the protein component of the encapsulating matrix. Preferably, the protein maintains a

significant portion of its original activity after having been processed to form microparticles

In another particularly preferred embodiment, at least a portion of the protein is immunoglobulins. These immunoglobulins may serve as a targeting agent. For
5 example, the binding site of the immunoglobulin may be directed to an epitope normally found in a tissue or on the cell surface of cells being targeted. The targeting of a specific receptor may lead to endocytosis of the microparticle. For example, the antibody may be directed to the LDL receptor.

The protein component may be provided using any means known in the art. In
10 certain preferred embodiments, the protein is commercially available. The protein may also be purified from natural or recombinant sources, or may be chemically synthesized. In certain preferred embodiments, the protein has been purified and is 75% pure, more preferably 90% pure.

15 *Sugar*

The sugar component of the LPSPs may be any simple or complex sugar. The sugar component of the matrix is thought to play a structural role in the particles and may also lead to increased biocompatibility. The percent of sugar in the LPSP matrix excluding the agent can range from 0% to 99%, more preferably from approximately
20 0.5% to approximately 50%, and most preferably from approximately 0.5% to approximately 40%.

Natural as well as unnatural sugars may be used in the inventive LPSPs. Sugars that may be used in the present invention include, but are not limited to, galactose, lactose, glucose, maltose, starches, cellulose and its derivatives (e.g.,

methyl cellulose, carboxymethyl cellulose, *etc.*), fructose, dextran and its derivatives, raffinose, mannitol, xylose, dextrans, glycosaminoglycans, sialic acid, chitosan, hyaluronic acid, and chondroitin sulfate. Preferably, the sugar component like the protein and lipid components is biocompatible and biodegradable. In certain

5 preferred embodiment, the sugar component is a mixture of sugars. The sugar may be from natural sources or may be synthetically prepared. Preferably, the sugar is available commercially.

In a particularly preferred embodiment, the sugar of the matrix may also function as a targeting agent. For example, the ligand of a receptor found on the cell

10 surface of cells being targeted or a portion of the ligand may be the same sugar in the LPSP or may be similar to the sugar in the LPSP, or the sugar may also be designed to mimic the natural ligand of the receptor.

Targeting Agents

15 The inventive LPSPs may be modified to include targeting agents since it is often desirable to target a LPSP to a particular cell, collection of cells, or tissue. A variety of targeting agents that direct pharmaceutical compositions to particular cells are known in the art (see, for example, Cotten *et al. Methods Enzym.* 217:618, 1993; incorporated herein by reference). The targeting agents may be included throughout

20 the particle or may be only on the surface. The targeting agent may be a protein, peptide, carbohydrate, glycoprotein, lipid, small molecule, *etc.* The targeting agent may be used to target specific cells or tissues or may be used to promote endocytosis or phagocytosis of the particle. Examples of targeting agents include, but are not limited to, antibodies, fragments of antibodies, low-density lipoproteins (LDLs),

transferrin, asialycoproteins, gp120 envelope protein of the human immunodeficiency virus (HIV), carbohydrates, receptor ligands, sialic acid, *etc.* If the targeting agent is included throughout the particle, the targeting agent may be included in the mixture that is spray dried to form the particles. If the targeting agent is only on the surface, the targeting agent may be associated with (*i.e.*, by covalent, hydrophobic, hydrogen bonding, van der Waals, or other interactions) the formed particles using standard chemical techniques.

10 *Pharmaceutical Compositions*

Once the LPSPs have been prepared, they may be combined with other pharmaceutical excipients to form a pharmaceutical composition. As would be appreciated by one of skill in this art, the excipients may be chosen based on the route of administration as described below, the agent being delivered, time course of delivery of the agent, *etc.*

Pharmaceutical compositions of the present invention and for use in accordance with the present invention may include a pharmaceutically acceptable excipient or carrier. As used herein, the term "pharmaceutically acceptable carrier" means a non-toxic, inert solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. Some examples of materials which can serve as pharmaceutically acceptable carriers are sugars such as lactose, glucose, and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as sodium carboxymethyl cellulose, ethyl cellulose, and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients such as cocoa butter and

suppository waxes; oils such as peanut oil, cottonseed oil; safflower oil; sesame oil; olive oil; corn oil and soybean oil; glycols such as propylene glycol; esters such as ethyl oleate and ethyl laurate; agar; detergents such as Tween 80; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free

5 water; isotonic saline; Ringer's solution; ethyl alcohol; and phosphate buffer solutions, as well as other non-toxic compatible lubricants such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, releasing agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the composition, according to the judgment of the formulator. The

10 pharmaceutical compositions of this invention can be administered to humans and/or to animals, orally, rectally, parenterally, intracisternally, intravaginally, intranasally, intraperitoneally, topically (as by powders, creams, ointments, or drops), buccally, or as an oral or nasal spray.

Liquid dosage forms for oral administration include pharmaceutically

15 acceptable emulsions, microemulsions, solutions, suspensions, syrups, and elixirs. In addition to the active ingredients (*i.e.*, LPSPs), the liquid dosage forms may contain inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol,

20 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and perfuming agents.

Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution, suspension, or emulsion in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, U.S.P. and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid are used in the preparation of injectables. In a particularly preferred embodiment, the LPSPs are suspended in a carrier fluid comprising 1% (w/v) sodium carboxymethyl cellulose and 0.1% (v/v) Tween 80.

The injectable formulations can be sterilized, for example, by filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

Compositions for rectal or vaginal administration are preferably suppositories which can be prepared by mixing the LPSPs with suitable non-irritating excipients or carriers such as cocoa butter, polyethylene glycol, or a suppository wax which are solid at ambient temperature but liquid at body temperature and therefore melt in the rectum or vaginal cavity and release the microparticles.

Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the LPSPs are mixed with at least one inert, pharmaceutically acceptable excipient or carrier such as sodium citrate or

- dicalcium phosphate and/or a) fillers or extenders such as starches, lactose, sucrose, glucose, mannitol, and silicic acid, b) binders such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidone, sucrose, and acacia, c) humectants such as glycerol, d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate, e) solution retarding agents such as paraffin, f) absorption accelerators such as quaternary ammonium compounds, g) wetting agents such as, for example, cetyl alcohol and glycerol monostearate, h) absorbents such as kaolin and bentonite clay, and i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof. In the case of capsules, tablets, and pills, the dosage form may also comprise buffering agents.

Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

- The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They may optionally contain opacifying agents and can also be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes.

Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

Dosage forms for topical or transdermal administration of an inventive pharmaceutical composition include ointments, pastes, creams, lotions, gels, powders, solutions, sprays, inhalants, or patches. The LPSPs are admixed under sterile conditions with a pharmaceutically acceptable carrier and any needed preservatives or buffers as may be required. Ophthalmic formulation, ear drops, and eye drops are also contemplated as being within the scope of this invention.

The ointments, pastes, creams, and gels may contain, in addition to the LPSPs of this invention, excipients such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc, and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to the LPSPs of this invention, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates, and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants such as chlorofluorohydrocarbons.

Transdermal patches have the added advantage of providing controlled delivery of a compound to the body. Such dosage forms can be made by dissolving or dispensing the LPSPs in a proper medium. Absorption enhancers can also be used to increase the flux of the compound across the skin. The rate can be controlled by either providing a rate controlling membrane or by dispersing the LPSPs in a polymer matrix or gel.

Methods of Making Microparticles

The inventive microparticles may be prepared using any method known in this art. These include spray drying, single and double emulsion solvent evaporation,

solvent extraction, phase separation, simple and complex coacervation, and other methods well known to those of ordinary skill in the art. A particularly preferred method of preparing the particles is spray drying. The conditions used in preparing the microparticles may be altered to yield particles of a desired size or property (e.g.,
5 hydrophobicity, hydrophilicity, external morphology, "stickiness", shape, etc.). The method of preparing the particle and the conditions (e.g., solvent, temperature, concentration, air flow rate, etc.) used may also depend on the agent being encapsulated and/or the composition of the matrix.

Methods developed for making microparticles for delivery of encapsulated
10 agents are described in the literature (for example, please see Doubrow, M., Ed., "Microcapsules and Nanoparticles in Medicine and Pharmacy," CRC Press, Boca Raton, 1992; Mathiowitz and Langer, *J. Controlled Release* 5:13-22, 1987; Mathiowitz *et al. Reactive Polymers* 6:275-283, 1987; Mathiowitz *et al. J. Appl. Polymer Sci.* 35:755-774, 1988; each of which is incorporated herein by reference).

15 If the particles prepared by any of the above methods have a size range outside of the desired range, the particles can be sized, for example, using a sieve.

As mentioned above, LPSPs are preferably prepared by spray drying. Prior methods of spray drying, such as those disclosed in PCT WO 96/09814 by Sutton and Johnson (incorporated herein by reference), provide the preparation of smooth,
20 spherical microparticles of a water-soluble material with at least 90% of the particles possessing a mean size between 1 and 10 micrometers. The method disclosed by Edwards *et al.* in U.S. Patent 5,985,309 (incorporated herein by reference) provides rough (non-smooth), non-spherical microparticles that include a water-soluble material combined with a water-insoluble material. Any of the methods described

above may be used in preparing the inventive LPSPs. Specific methods of preparing LPSPs containing bupivacaine are described below in the Examples.

Administration

- 5 The LPSPs and pharmaceutical compositions containing LPSPs may be administered to an individual via any route known in the art. These include, but are not limited to, oral, sublingual, nasal, intradermal, subcutaneous, intramuscular, rectal, vaginal, intravenous, intraarterial, and inhalational administration. As would be appreciated by one of skill in this art, the route of administration is determined by
- 10 the agent being administered, the target organ, the preparation being administered, time course of administration, disease being treated, *etc.*

- In one particularly preferred embodiment, LPSPs containing a local anesthetic (*e.g.*, bupivacaine, lidocaine, mepivacaine) are administered in the vicinity of a nerve to provide a nerve block. Nerve blocks provide a method of anesthetizing large areas
- 15 of the body without the risks associated with general anesthesia. Any nerve may be anesthetized in this manner. The LPSPs containing the agent are deposited as close to the nerve as possible without injecting directly into the nerve. Particularly preferred nerves include the sciatic nerve, the femoral nerve, inferior alveolar nerve, nerves of the brachial plexus, intercostal nerves, nerves of the cervical plexus, median nerve,
- 20 ulnar nerve, and sensory cranial nerves. In a particularly preferred embodiment, epinephrine or another vasoactive agent is administered along with the local anesthetic to prolong the block. The epinephrine or other agent (*e.g.*, other vasoactive agents, steroidal compounds, non-steroidal anti-inflammatory compounds) may be encapsulated in the LPSPs containing the local anesthetic, encapsulated in LPSPs by

itself, or unencapsulated. One of ordinary skill in this art would be able to determine the choice of local anesthetic as well as the amount and concentration of anesthetic based on the nerves and types of nerve fibers to be blocked, the duration of anesthesia required, and the size and health of the patient (Hardman & Limbird, Eds., *Goodman & Gilman's The Pharmacological Basis of Therapeutics Ninth Edition*, Chapter 15, pp. 331-347, 1996; incorporated herein by reference).

These and other aspects of the present invention will be further appreciated upon consideration of the following Examples, which are intended to illustrate certain particular embodiments of the invention but are not intended to limit its scope, as defined by the claims.

Examples

Example 1-Sciatic Nerve Blockade with Lipid-Protein-Sugar Particles Containing Bupivacaine

In this Example, the production and characterization of LPSPs *in vitro* is discussed along with the assessment of the *in vivo* local anesthetic efficacy of an optimal formulation in sciatic nerve blockade in the rat, using a neurobehavioural paradigm (Kohane *et al.* "A re-examination of tetrodotoxin for prolonged anesthesia" *Anesthesiology* 89:119-131, 1998; Thalhammer *et al.* "Neurologic evaluation of the rat during sciatic nerve block with lidocaine" *Anesthesiology* 82:1013-1025, 1995; each of which is incorporated herein by reference) that examines sensory (thermal

nociception) and motor (weight bearing) function. The marked methodological variability between published reports on the *in vivo* effectiveness of controlled release local anesthetic preparations makes comparisons between them difficult. For this reason the LPSP delivery system is compared to another delivery system in a series of

5 experiments where the particles are delivered by the same means, to the same location, in equal quantity, and where the neurobehavioural outcomes are evaluated by the same methods and at the same time intervals in a blinded manner. Large (20 to 120 μm) poly(lactic-co-glycolic) acid (PLGA)-based particles have been selected for this comparison because a) they have a long track-record of experimental use for

10 anesthesia in the peripheral nervous system and spinal cord (Castillo *et al.* "Glucocorticoids prolong rat sciatic nerve blockade *in vivo* from bupivacaine microspheres" *Anesthesiology* 85:1157-1166, 1996; Curley *et al.* "Prolonged regional nerve blockade. Injectable biodegradable bupivacaine/polyester microspheres" *Anesthesiology* 84:1401-1410, 1996; Drager *et al.* "Prolonged intercostal nerve

15 blockade in sheep using controlled release bupivacaine and dexamethasone from polyester microspheres" *Anesthesiology* 89:969-979, 1998; Estebe *et al.* "Prolongation of spinal anesthesia with bupivacaine-loaded (DL-lactide) microspheres" *Anesth. Analg.* 81:99-103, 1995; Le Corre *et al.* "Preparation and characterization of bupivacaine-loaded polylactide and polylactide-coglycolide

20 microspheres" *Int. J. Pharmaceut.* 107:41-49, 1994; Le Corre *et al.* "In vitro controlled release kinetics of local anaesthetics from poly(D,L-lactide) and poly(lactide-co-glycolide) microspheres" *J. Microencaps.* 14:243-255, 1997; Wakiyama *et al.* "Preparation and evaluation in vitro of polylactic acid microspheres containing local anesthetics" *Chem. Pharm. Bull.* 29:3363-3368, 1981; Wakiyama *et*

- al.* "Preparation and evaluation *in vitro* and *in vivo* of polylactic acid microspheres containing dibucaine" *Chem. Pharm. Bull.* 30:3719-3727, 1982; each of which is incorporated herein by reference), and b) such microspheres have been described as producing very slow release of local anesthetics (Curley *et al.* "Prolonged regional nerve blockade. Injectable biodegradable bupivacaine/polyester microspheres" *Anesthesiology* 84:1401-1410, 1996; incorporated herein by reference).

Materials and Methods

Materials

- Bupivacaine hydrochloride, human serum albumin (Fraction V), and lactose β -monohydrate were purchased from Sigma Chemical Co. (St. Louis, MO), L- α -dipalmitoylphosphatidylcholine (DPPC) from Avanti Polar Lipids (Alabaster, AL), poly (lactic-co-glycolic) acid (65:35, MW 110,000) (PLGA) from Medisorb, poly (vinyl alcohol) (88% hydrolyzed, MW 20,000) from Polysciences (Warrington, PA), ethyl acetate and methylene chloride (both HPLC grade) from EM Sciences (Gibbstown, NJ), and USP grade ethanol from Pharmco Products, Brookfield, CT. Bupivacaine hydrochloride was made into the free base by alkaline precipitation and filtration. The ultraviolet absorbance spectrum from 200 nm to 300 nm, and a standard curve of absorbance at 272 nm vs. concentration were determined for each batch of the free base for quality control purposes.

Preparation of spray-dried lipid-protein particles (LPSPs)

A 70:30 (v/v) ethanol:water solvent system was employed for solubilization and spray drying of excipients and bupivacaine. The solutions were prepared in the following manner: (i) the DPPC and bupivacaine free base were dissolved in a given

amount of ethanol, (ii) the lactose and albumin were dissolved in a given amount of water (pH adjusted to 7.0), and (iii) the solutions were mixed immediately prior to spray drying. Solute concentrations ranged from 1 to 4 grams per liter. The proportion of albumin to lactose was kept constant in experiments where the DPPC content was changed.

Solutions were spray-dried using a Model 190 bench top spray drier (Büchi Co, Switzerland). The spray-drying parameters (inlet temperature, fluid flow rate, drying airflow rate, and aspirator pressure) were optimized based on the yield and size characteristics of both the blank (no bupivacaine) and the bupivacaine-containing particles. The optimized conditions were: inlet temperature = 115 to 120°C, solution feed rate = 12 to 14 ml/min, drying airflow rate = 600 l/min, and aspirator pressure = 18 barr. These conditions typically resulted in outlet temperatures in the range of 50 to 55°C for a given experimental run.

Particle size and bulk density

A small amount of particles was dispersed in 20 ml of Isotoner (Coulter Corp., Miami, FL) and analyzed for size via a Coulter Multisizer (Coulter Electronics Ltd., Luton, U.K.) equipped with a 50 µm (for LPSPs) or 140 µm (for PLGA microspheres) orifice tube. Bulk mass density of the 60% DPPC powders was estimated using a Dual Platform Microprocessor Controlled Tap Density Tester (Vankel Technology Group, Cary, NC).

Scanning electron microscopy

The surface morphology of spray-dried particles was examined using a JEOL Model 6320 FV field emission scanning electron microscope (provided by the Massachusetts Institute of Technology Department of Materials Science and

Engineering Electron Microscopy Center). Samples were mounted on stubs and coated with a layer of gold/palladium. Samples were scanned at voltages between 5 and 10 kV at a probe current setting of 3 and a working distance of 7 millimeters.

Bupivacaine content of LPSPs

- 5 In order to determine the bupivacaine content of LPSPs, 10 mg of particles were agitated (Touch Mixer model 2332, Fisher Scientific, Pittsburgh, PA) for 20 seconds in 1 ml ethyl acetate. One-half ml of 0.1 N NaOH was then added, and the mixture was agitated for an additional 60 seconds. The suspension was centrifuged for 10 minutes at 14,000 rpm. One half ml of the upper organic layer was withdrawn,
- 10 diluted with an equal volume of fresh ethyl acetate, and the absorbance at 272 nm was then measured (Cary 50 Bio UV-Visible Spectrophotometer, Varian, Australia) in a quartz cuvette (Hellma, Mullheim, Germany). Bupivacaine content was determined by comparison to a standard curve. Blank (no bupivacaine) LPSPs served as controls, and when processed in this manner had negligible absorbance at 272 nm. As an
- 15 additional control we determined the amount of albumin that may have accompanied the bupivacaine in the ethyl acetate extraction (this was important because the two compounds have overlapping absorbance spectra), using a commercial kit (BCA Protein Assay Reagent Kit, Pierce Chemical Co., Rockford, IL). The amount of
- 20 albumin was below the detection limit ($< 25 \mu\text{g/ml}$), and therefore could not account for measured absorbances at 272 nm.

In vitro release of bupivacaine from microparticles

Fifty mg of LPSPs or PLGA microspheres were suspended in 1 ml phosphate buffered saline pH 7.4 at 37°C and inserted into the lumen of a Spectra/Por 1.1 Biotech Dispodialyzer with an 8,000 MW cut-off. The dialysis bag was placed into a

test tube with 12 ml PBS and incubated at 37°C on a tilt-table (Ames Aliquot Mixer, Miles). At predetermined intervals, the dialysis bag was transferred to a test tube with fresh PBS. The bupivacaine concentration in the dialysate was quantitated by measuring absorbance at 272 nm and referring to a standard curve. Observation of
5 the entire spectrum, and performance of a protein assay (as above) confirmed the absence of albumin from the samples that were measured.

Preparation and characterization of PLGA-bupivacaine microspheres

Microspheres loaded with 10% (w/w) and 50% (w/w) bupivacaine were prepared using a single emulsion method (Curley *et al.* "Prolonged regional nerve
10 blockade. Injectable biodegradable bupivacaine/polyester microspheres" *Anesthesiology* 84:1401-1410, 1996; Watts *et al.* "Microencapsulation using emulsification/solvent evaporation: an overview of techniques and applications" *Crit. Rev. Ther. Drug Carr. Sys.* 7:235-259, 1990; each of which is incorporated herein by reference). Bupivacaine and PLGA were dissolved in methylene chloride, and the
15 mixture was homogenized (Silverson L4R, Silverson Machines Ltd., Cheshire, England) in 50 ml 0.5% polyvinyl alcohol in 100 mM Trizma buffer pH 8.5 for 60 seconds. The resulting suspension was decanted into 100 ml of 0.05 % polyvinyl alcohol in 100 mM Trisma pH 8.5 and stirred for 3 minutes prior to rotary evaporation (Büchi Rotavap, Büchi, Switzerland) in a 37°C water bath until bubbling ceased.
20 Spheres 20 µm to 120 µm in diameter were isolated by sieving (Newark Wire Co., Newark, NJ), then resuspended in 50 ml of water. The suspension was washed three times by centrifugation at 5000 rpm for 5 minutes. The final pellet was lyophilized to dryness.

Bupivacaine content was determined by dissolving 10 mg of microspheres in 1

ml methylene chloride, and comparing the resulting UV absorbance at 272 nm to a standard curve. Under similar conditions, PLGA microspheres containing no bupivacaine showed negligible absorbance at 272 nm.

Animal Care

- 5 Young adult male Sprague-Dawley rats weighing 310 - 420 g each were obtained from Taconic Farms (Germantown, NY), and housed in groups in a 6 AM - 6 PM light-dark cycle. Animals were cared for in compliance with protocols approved by the Animal Care and Use Committee at the Massachusetts Institute of Technology, and the Principles of Laboratory Animal Care published by the National
10 Institutes of Health. Rats were only injected once.

Sciatic Blockade Technique

- Prior to nerve block injections, rats were anesthetized briefly (< 2 minutes) with halothane. Concurrently, 75 mg of LPSPs or microspheres were suspended in 0.6 ml of carrier fluid (1% (w/v) sodium carboxymethyl cellulose, 0.1% (v/v) Tween
15 80) with gentle agitation (< 5 sec) in preparation for injection. A 20G needle was introduced postero-medial to the greater trochanter, pointing in an anteromedial direction (Thalhammer *et al.* "Neurologic evaluation of the rat during sciatic nerve block with lidocaine" *Anesthesiology* 82:1013-1025, 1995; incorporated herein by reference). Once bone was contacted, the needle was withdrawn 1 mm and the
20 particle-containing solution was injected. The left leg was always used for blocks; the right served as control.

Assessment of Nerve Blockade

 The effectiveness of block was measured at various time points, applying the methods of Thalhammer *et al.* (Thalhammer *et al.* "Neurologic evaluation of the rat

during sciatic nerve block with lidocaine" *Anesthesiology* 82:1013-1025, 1995; incorporated herein by reference), or modifications thereof (Kohane *et al.* "A re-examination of tetrodotoxin for prolonged anesthesia" *Anesthesiology* 89:119-131, 1998; incorporated herein by reference).

- 5 Nociceptive block was assessed by a modified hotplate test (Masters *et al.* "Prolonged regional nerve blockade by controlled release of local anesthetic from a biodegradable polymer matrix" *Anesthesiology* 79:1-7, 1993; incorporated herein by reference). Hind paws were exposed in sequence (left then right) to a hot plate at 56°C (Model 39D Hot Plate Analgesia Meter, IITC Inc., Woodland Hills, CA), and
- 10 the time (latency) until paw withdrawal was measured by a stopwatch. Thermal latency is a measure of the intensity of analgesia. If the paw remained in contact for 12 seconds, it was removed by the experimenter to avoid injury to the animal or the development of hyperalgesia. This test was repeated three times for each rat at every time-point.

- 15 Motor strength was assessed by holding the rat with its posterior above a digital balance and allowing it to bear weight on one hind paw at a time. The maximum weight that the rat could bear without its ankle touching the balance was quantified.

Neurobehavioural Data Processing

- 20 The data for nociceptive block are reported in terms of thermal latency (intensity) and duration of block. The duration of thermal nociceptive block is the time required for thermal latency to return to a value of 7 seconds (which is 50% of maximal block when a baseline thermal latency of approximately 2 seconds is taken into account). The duration of motor block was defined as the time for weight bearing

to return halfway to normal from maximal block.

Statistical Analysis

Data are reported as means with standard deviations. Comparisons between groups were made using Student's t-test. These tests were unpaired except when

5 comparing sensory vs. motor blockade in the same rat.

Results

Production and characterization of Lipid-Protein-Sugar Particles (LPSPs)

The spray-drying process conditions were initially optimized (with respect to
10 yield) for the production of blank excipient particles (60:20:20
DPPC:albumin:lactose), as discussed in the methods section. (The reported
percentage of DPPC refers to the composition of the excipients, excluding the
delivered drug.) These conditions also appeared to be satisfactory for the production
of the 10% (w/w) bupivacaine particles with varying DPPC contents. The results
15 obtained from typical runs are shown in Table 1.

Table 1. Characteristics of lipid-protein-sugar particles (LPSPs) and PLGA-based microspheres

Micro-particle	DPPC (%) ^a	Bupivacaine loading (%)	n	Yield ^{b, c} (%)	Median diameter ^{b, d} (μm)	Bupivacaine ^{b, e} (%)
LPSP	3	10	5	40 ± 6	2.58 ± 0.22	8 ± 0.4
	60	0	5	25 ± 5	4.66 ± 0.33	-
	60	10	5	37 ± 3	4.44 ± 0.39	8.8 ± 0.7
	99	10	5	37 ± 7	1.73 ± 0.05	7.6 ± 0.8

PLGA micro- sphere	0	50	6	53 ± 15	59 ± 12	44 ± 5
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- a. Percentage of the total mass of excipient.
- b. Values indicated are means ± standard deviations.
- c. The fraction of solutes recovered, as weight % of the total amount of solutes taken in preparation.
- 5 d. Volume-weighted.
- e. Measured as described in Methods (above).

The volume of fifty milligrams of each formulation loaded into test tubes were measured, and their densities were calculated. The 60% DPPC particles were less

10 dense (0.07 ± 0.004 g/ml) than the 3% DPPC (0.24 ± 0.025 , $p = 0.0007$) and 99% DPPC (0.14 ± 0.017 , $p = 0.003$) particles. This difference in density was probably due to a difference in particle size (Table 1): the mean diameter of the 60% DPPC particles was greater than those of the 3% and 99% DPPC particles ($p = 0.00001$ and 0.000006 respectively). The 3% and 99% DPPC particles consistently formed

15 macroscopic balls when stored as a dry powder, while this was not seen with the 60% DPPC particles. The impression of aggregation was confirmed by the observation that the average diameter of 3% and 99% DPPC particles rose to 19.28 ± 0.01 and 11.08 ± 0.34 μm respectively over a period of 4 weeks storage in a dessicator, while those of 60% DPPC particles did not change. The bupivacaine content of the various

20 LPSPs formulations was similar ($p = \text{n.s.}$).

The 60% DPPC particles were spheroidal or, as can be seen in Fig. 1, concave in shape. Typical observed diameters were in the range of 3 to 5 microns. To ensure that the structural integrity of the particles was not impaired by the delivery method, we suspended 10 mg of particles in the carrier fluid (1% (w/v) carboxymethyl

25 cellulose, 0.1% (v/v) Tween 80), mechanically agitated them for 10 seconds then

injected them through a 20 G needle onto the inner wall of a test tube. Scanning electron microscopy demonstrated that the structure of the LPSPs was not altered (not shown).

Production and characterization of PLGA-Bupivacaine microspheres

- 5 PLGA microspheres produced as described in the methods section appeared to be regular spheres as viewed by light microscopy (images not shown), with diameters more than ten times larger than those of the LPSPs. Data from microsphere production are in Table 1. The final particle yield was comparable to that of the spray-dried particles. The data relevant to the production of the 10% (w/w)
- 10 bupivacaine microspheres were similar to those for the 50% (w/w) microspheres, and their mean bupivacaine content (w/w) was 8 % (n = 2).

Bupivacaine release from LPSPs

These experiments were performed so as to allow rational selection of a particular formulation for use in *in vivo* studies.

- 15 Pilot studies had shown that 3% DPPC particles almost completely disappeared by 18 hours after suspension in phosphate buffered saline, while 60% and 99% particles lasted many days. Consequently, we focused on the latter preparations. Bupivacaine release from 50 mg samples of 10% loaded (w/w) bupivacaine-LPSPs (n = 4 for each particle formulation) was measured. Figure 2 shows the cumulative
- 20 release of bupivacaine over time. Both particle types caused delayed release of bupivacaine into the dialysate compared to the unencapsulated drug (1 ml of 0.5% (w/v) bupivacaine, or 5 mg). Both 60% and 99% DPPC particles completely released their bupivacaine content within 24 hours. However, release from the 60% DPPC particles was more gradual: at 9 hours, the 60% DPPC particles had released $53.8 \pm$

1.5 % of their bupivacaine content, whereas the 99% DPPC particles had released 80.6 ± 4.7 % ($p = 0.0002$). Consequently, the 60% DPPC formulation was selected for *in vivo* studies. Figure 2 also shows the release of bupivacaine from 50% (w/w) PLGA particles ($n = 4$). The release, on a percentage basis, was much slower than
5 that from LPSPs: less than 50% of total drug content was released in 30 hours, at which time the LPSPs had released 100% of drug content. The total amount of drug released by the LPSPs was slightly greater than that released by PLGA microspheres at most early time points (by 3.5 hours, the LPSPs had released 1.65 ± 0.17 mg of bupivacaine vs. 1.26 ± 0.15 mg for PLGA microspheres, $p = 0.01$). This relationship
10 was reversed at longer durations (by 9 hours the LPSPs had released 2.6 ± 0.2 mg of bupivacaine, compared to 4.2 ± 0.7 mg for the PLGA microspheres ($p = 0.02$)).

Effectiveness of sciatic nerve block

Rats were injected at the sciatic nerve with 75 mg (≈ 215 mg/kg) of spray-dried LPSPs containing 10% (w/w) bupivacaine, and the time course of nerve
15 blockade was followed. All rats injected with 10% (w/w) bupivacaine LPSPs achieved maximal nerve block (thermal latency = 12 seconds) by the time of the first testing (30 minutes). Four out of ten rats injected with 50% bupivacaine microspheres did not achieve maximal block by that time. Nine out of ten rats injected with 50% (w/w) bupivacaine microspheres had maximal block by one hour after injection. All
20 achieved maximal block within 3 hours.

The average duration of thermal nociceptive block from 10% (w/w) bupivacaine LPSPs was 468 ± 210 min ($n = 10$). The duration of thermal nociceptive block obtained from injection with 75 mg of PLGA microspheres with 50% (w/w) loading of bupivacaine was 706 ± 344 min ($n = 10$). This was not statistically

different from the duration obtained with the 10% (w/w) bupivacaine LPSPs ($p = 0.08$).

In order to compare the efficacy of equal loading with bupivacaine, rats were injected with 75 mg of 10 % (w/w) bupivacaine PLGA microspheres ($n = 5$), and 50% (w/w) bupivacaine LPSPs ($n = 2$). The former did not result in nerve block as defined by our paradigm, while the latter caused rapid demise of the rats.

Six (6) rats were injected with 75 mg of blank DPPC-albumin-lactose particles in order to verify that the increased efficiency (comparable duration of block with much lower drug loading) of the LPSPs over bupivacaine microspheres was not due to an intrinsic nerve blocking-effect of the component excipients. Blank LPSPs did not produce any detectable nerve block.

Modality-specificity of nerve blockade.

Blank LPSPs and 10% (w/w) bupivacaine microspheres did not cause any impairment in sensory or motor function. Motor blockade from 10% (w/w) bupivacaine LPSPs lasted 508 ± 258 min, while that from 50% (w/w) bupivacaine microspheres lasted 1062 ± 456 min ($p = 0.005$). Fig. 3 focuses on the clinically important comparison of the durations of motor block (x-axis) and sensory block (y-axis) for both preparations. The motor block from the PLGA microsphere preparation lasted 50% longer than did the sensory block ($p = 0.003$), as evidenced by the location of the representative point below the line of unity. The LPSPs had durations of sensory and motor block that were not statistically different from each other (8% difference, $p = 0.37$).

Systemic distribution of bupivacaine.

The presence of functional deficits in the un-injected extremity was used as a

measure of the degree of systemically distributed local anesthetic (*i.e.*, toxicity).

Thermal latency (the length of time that a rat would leave his paw on the hotplate) was measured in the un-injected leg at predetermined intervals, in rats who received 10% bupivacaine LPSPs or 50% bupivacaine microspheres (Fig. 4). There was no statistically significant difference between the mean latencies in the two groups at any time point.

One rat (out of 11) injected with 50% bupivacaine microspheres died, approximately 2 hours after injection. Necropsy revealed congestion of the liver and kidneys, most consistent with heart failure. Both rats injected with 50% (w/w) bupivacaine LPSPs died. There were no deaths in the 10% (w/w) bupivacaine LPSP group (n = 10), or 10% (w/w) PLGA microsphere group.

Encapsulation improved the safety and efficacy of bupivacaine. None of the rats injected with 10% (w/w) bupivacaine LPSPs had marked increases in contralateral latency. In comparison, rats (n = 6) injected with an equivalent amount of bupivacaine in solution (1.5 ml of 0.5% bupivacaine, *i.e.* 7.5 mg) had a duration of block of 166 ± 55 min. For this experiment larger rats (approx. 410 g) were used than those used in the remainder of the study, in order to avoid animal death (the median lethal dose of bupivacaine in adult rats is 30 ± 5 mg/kg (Kohane *et al.* "Sciatic nerve blockade in infant, adolescent, and adult rats: a comparison of ropivacaine and bupivacaine" *Anesthesiology* 89:1199-1208, 1998; incorporated herein by reference), or 10.5 mg in a 350 g rat). Even so, one of those rats had severe signs of systemic toxicity (thermal latency = 12 seconds in the uninjected leg). It was not possible to directly compare the efficacy of bupivacaine solution and 50% bupivacaine microspheres, since the dose of bupivacaine contained in 75 mg of those microspheres

(38.5 mg) is approximately three times the median lethal dose of the unencapsulated drug (Kohane *et al.* "Sciatic nerve blockade in infant, adolescent, and adult rats: a comparison of ropivacaine and bupivacaine" *Anesthesiology* 89:1199-1208, 1998). Nevertheless, it is obvious that the microspheres increased the safety of bupivacaine.

5

Discussion

Of the three LPSP formulations tested *in vitro*, the 60% DPPC particles appeared optimal in terms of drug release of bupivacaine. The slower release of bupivacaine from the 60% DPPC particles compared to the 99% DPPC particles was somewhat surprising; *a priori* one might have expected the more hydrophobic nature of the latter particle type to delay release to a greater extent. This discrepancy may be related to the larger size of the 60% DPPC particles, which may impede access of water to the encapsulated drug and of drug to the exterior, or to a degree of bupivacaine binding by albumin.

15 The DPPC-albumin-lactose particles appear to be effective as vehicles for the local delivery of percutaneously injected local anesthetics in rats. The LPSPs had a more rapid onset of nerve block than the PLGA microspheres, which may be a reflection of the initial more rapid release of drug from the LPSPs. They were as effective as PLGA microspheres in terms of duration of local anesthesia, with one-fifth the initial loading of drug. (The duration of block that we obtained with the 50% bupivacaine microspheres is considerably longer than previously published values. Seventy-five percent loaded particles have been reported to last 6.0 ± 3.0 hours (Curley *et al.* "Prolonged regional nerve blockade. Injectable biodegradable bupivacaine/polyester microspheres" *Anesthesiology* 84:1401-1410, 1996;

20

incorporated herein by reference), compared to 11.8 ± 5.7 hours for the 50% loaded particles in this study.) It would appear, based on the *in vitro* release studies, that this improved ratio of duration of block to drug loading most likely stems from the proportionally more rapid release of drug from the LPSPs. An alternative explanation would be that the LPSPs themselves have an effect on nerve function. While this possibility cannot be excluded, LPSPs without bupivacaine did not cause any detectable deficits in nerve function.

The more rapid fractional release of drug from the LPSPs did not result in greater toxicity than occurred from PLGA microspheres, as evidenced by the fact that thermal latency in the uninjected leg was not increased. (Increases in contralateral latency are an early sign of severe local anesthetic toxicity (Kohane *et al.* "Sciatic nerve blockade in infant, adolescent, and adult rats: a comparison of ropivacaine and bupivacaine" *Anesthesiology* 89:1199-1208, 1998; incorporated herein by reference).) The *in vitro* data suggest that this was because the discrepancy in total drug release between the two particle types was not as great as the fractional (percentage) difference. PLGA microspheres would appear to provide a better margin of safety at high bupivacaine loadings.

In general, it is not desirable for the motor block to be of longer duration than the sensory block (resulting in a paralyzed limb with full sensation). In fact, there are applications (such as obstetric anesthesia) where sensory block in the absence of motor block is desirable (so the mother can push during labor while still obtaining pain relief). The LPSPs had a more favorable ratio of duration of sensory to motor block than did the PLGA microspheres. The explanation for this difference is most likely to be pharmacokinetic. The large myelinated fibers ("A fibers") that mediate

motor function are more sensitive to amino-amide local anesthetics than are the small unmyelinated fibers that mediate pain ("C fibers") (Wildsmith *et al.* "Differential nerve blocking activity of amino-ester local anaesthetics" *Br. J. Anaesth.* 57:612-620, 1985; incorporated herein by reference). Thus, one would expect sensation to return
5 before motor function. In the case of the PLGA microspheres, the rate of decline of the local concentration of bupivacaine is probably slower, so that the time interval between the termination of sensory blockade and motor blockade is longer. The kinetic argument for the difference between the functional selectivities of LPSPs and PLGA microspheres is supported by the observation that bupivacaine solution (in the
10 absence of any controlled release device) also shows approximately equal durations of sensory and motor block (Fig. 3), as has been previously noted in this animal model (Kohane *et al.* "Sciatic nerve blockade in infant, adolescent, and adult rats: a comparison of ropivacaine and bupivacaine" *Anesthesiology* 89:1199-1208, 1998; Kohane *et al.* "A re-examination of tetrodotoxin for prolonged anesthesia"
15 *Anesthesiology* 89:119-131, 1998; each of which is incorporated herein by reference).

It bears mentioning that the LPSPs are of a size and density that makes them suitable for inhalational delivery. (The tap density—a more standardized measure of particle density—of the 60% DPPC LPSPs was 0.11 ± 0.04 g/ml.) Nebulized local anesthetics have been used in a variety of roles in the management of medical
20 problems of the upper airway and pulmonary tree (Keane *et al.* "Comparison of nebulized and sprayed topical anaesthesia for fiberoptic bronchoscopy" *Eur. Respir. J.* 5:1123-1125, 1992; incorporated herein by reference), including the management of asthma (Decco *et al.* "Nebulized lidocaine in the treatment of severe asthma in children: a pilot study" *Ann. Allergy Asthma Immunol.* 82:29-32, 1999; Hunt *et al.*

“Effect of nebulized lidocaine on severe glucocorticoid-dependent asthma” *Mayo Clin. Proc.* 71:361-368, 1996; incorporated herein by reference). Nebulized lidocaine results in lower serum levels of drug than are achieved by equieffective intravenous doses (Groebe *et al.* “Both intravenous and inhaled lidocaine attenuate reflex bronchoconstriction but at different plasma concentrations” *Am. J. Respir. Crit. Care Med.* 159:530-535, 1999; incorporated herein by reference). Thus it is conceivable that particles of this sort could be beneficial in severe asthma, or in blunting patient responses to intratracheal suctioning, bronchoscopy, and other noxious procedures.

In summary, controlled release of bupivacaine using lipid-protein-sugar particles can provide prolonged duration local anesthesia that is as effective (depth and duration of anesthesia) as that conferred by conventional polymer-based particles.

Example 2 - Biocompatibility of Lipid-Protein-Sugar Particles Containing Bupivacaine in the Perineurium

In this second Example, the potential for a number of possible adverse reactions to the injected lipid-protein-sugar particles is assessed. The primary focus of the study was an examination of the biocompatibility of particles in terms of inflammatory response and gross neural injury as assessed by standard histological techniques. The incidence of “touch-evoked agitation” was also examined. This phenomenon was described in animals that received intrathecal injections of liposomes and phospholipid emulsions containing local anesthetics (Yanez *et al.* “Touch-evoked agitation produced by spinally administered phospholipid emulsion

- and liposomes in rats" *Anesthesiology* 82:1189-1198, 1995; incorporated herein by reference): they appeared in distress when the injected area was palpated. Although this phenomenon is not well understood, it is may be due to a product of phospholipid hydrolysis and to be particularly prominent in phospholipids whose gel-transition
- 5 temperatures are close to body temperature (Yanez *et al.* "Touch-evoked agitation produced by spinally administered phospholipid emulsion and liposomes in rats" *Anesthesiology* 82:1189-1198, 1995; incorporated herein by reference). Because of the unknown potential for nerve injury from these particles, the rats were also tested for the development of self-mutilation ("autotomy") in the blocked leg (Wall *et al.*
- 10 "Autotomy following peripheral nerve lesions: experimental anaesthesia dolorosa" *Pain* 7:103-111, 1979; incorporated herein by reference), a behavior that results from nerve injury and is believed to be pain-related (although this is controversial). PLGA microspheres *per se* do not produce autotomy (Xiao *et al.* "Prolonged sciatic nerve blockade prevents neither the rise in GAP-43 expression nor the development of pain-
- 15 related behaviour following nerve crush injury" *Anesthesiology* 87:734A, 1997; incorporated herein by reference) when injected at the perineurium.

Evaluation of the relative biocompatibility of different controlled release preparations described in the literature is impeded by the marked heterogeneity of experimental designs. Therefore, we compare LPSP to another delivery vehicle that

20 is commonly employed to deliver local anesthetics, microspheres made from high molecular weight poly(lactic-co-glycolic acid (PLGA) (Castillo *et al.* "Glucocorticoids prolong rat sciatic nerve blockade in vivo from bupivacaine microspheres" *Anesthesiology* 85:1157-66, 1996; Curley *et al.* "Prolonged regional nerve blockade. Injectable biodegradable bupivacaine/polyester microspheres"

- Anesthesiology* 84:1401-1410, 1996; Drager *et al.* "Prolonged intercostal nerve blockade in sheep using controlled release bupivacaine and dexamethasone from polyester microspheres" *Anesthesiology* 89: 969-979, 1998; Le Corre *et al.* "Preparation and characterization of bupivacaine-loaded polylactide and polylactide-coglycolide microspheres" *Int. J. Pharmaceut.* 107:41-49, 1994; Le Corre *et al.* "In vitro controlled release kinetics of local anaesthetics from poly(D,L-lactide) and poly(lactide-co-glycolide) microspheres" *J. Microencaps.* 14:243-255, 1997; Estebe *et al.* "Prolongation of spinal anesthesia with bupivacaine-loaded (DL-lactide) microspheres" *Anesth. Analg.* 81:99-103, 1995; Wakiyama *et al.* "Preparation and evaluation in vitro of polylactic acid microspheres containing local anesthetics" *Chem. Pharm. Bull.* 29:3363-3368, 1981; Wakiyama *et al.* "Preparation and evaluation in vitro and in vivo of polylactic acid microspheres containing dibucaine" *Chem. Pharm. Bull.* 30:3719-3727, 1982; each of which is incorporated herein by reference), in a blinded study. This comparison is particularly valuable since the biocompatibility and inflammatory potential of α -hydroxy acids such as PLGA (Cutright *et al.* "Histologic comparison of polylactic and polyglycolic acid sutures" *Oral Surg.* 32:165-173, 1971; Athanasiou *et al.* "Sterilization, toxicity, biocompatibility and clinical applications of polylactic acid/polyglycolic acid copolymers" *Biomaterials* 17:93-102, 1996; Brazeau *et al.* "Evaluation of PLGA microsphere size effect on myotoxicity using the isolated rodent skeletal muscle model" *Pharm. Dev. Technol.* 1:279-283, 1996; van der Elst *et al.* "Bone tissue response to biodegradable polymers used for intramedullary fracture fixation: A long-term in vivo study in sheep femora" *Biomaterials* 20: 121-128, 1999; each of which is incorporated herein by reference) when applied perineurally (Drager *et al.* "Prolonged

intercostal nerve blockade in sheep using controlled release bupivacaine and dexamethasone from polyester microspheres" *Anesthesiology* 89: 969-979, 1998; incorporated herein by reference) has been described. As many parameters as possible were kept constant between the groups (means and site of drug administration, weight of particle delivered per rat, behavioral observer, dissection and histological techniques and time intervals), and observations were made in a blinded manner.

Materials and Methods

10 *Materials*

Bupivacaine hydrochloride, human serum albumin (Fraction V), and lactose β -monohydrate were purchased from Sigma Chemical Co. (St. Louis, MO), L- α -dipalmitoylphosphatidylcholine (DPPC) from Avanti Polar Lipids (Alabaster, AL), poly (lactic-co-glycolic) acid (lactide:glycolide = 65:35, MW 110,000) (PLGA₁₁₀) from Alkermes (Cambridge, MA), poly (lactic-co-glycolic) acid (lactide:glycolide = 50:50, MW 20,000) (PLGA₂₀) from Boehringer Ingelheim (Ingelheim, Germany), poly (vinyl alcohol) (88% hydrolyzed, MW 20,000) from Polysciences (Warrington, PA), and USP grade ethanol from Pharmco Products (Brookfield, CT). Bupivacaine hydrochloride was made into the free base by alkaline precipitation and filtration.

20 *Preparation of spray-dried lipid-protein particles (LPSP) and PLGA microspheres*

LPSPs and PLGA microspheres were prepared and characterized (Kohane *et al.* "Sciatic nerve blockade with lipid-protein-sugar particles containing bupivacaine" *Pharm. Res.* 2000 (in press); incorporated herein by reference). In brief, LPSP were produced as follows. Dipalmitoylphosphatidyl-choline (DPPC) and bupivacaine free

- base were dissolved in ethanol, and albumin and lactose were dissolved in water. The two solution were mixed (so the final proportion (w/w) of solutes was DPPC 54: albumin 18: lactose 18: bupivacaine 10), and spray-dried using a Model 190 bench top spray drier (Büchi Co, Switzerland). PLGA microspheres containing 50% and 0% (w/w) bupivacaine were prepared by the single emulsion method using PLGA₁₁₀. Polymer and bupivacaine free base (200 mg total mass) were dissolved in 1.5 ml methylene chloride, and added to a solution of 1% polyvinyl alcohol in 100 mM Trizma buffer pH 8.5. The mixture was homogenized (Silverson L4R, Silverson Machines Ltd., Cheshire, England) at 3000 rpm, and methylene chloride was removed by rotary evaporation (Büchi Rotavap, Büchi, Switzerland) at 37 °C. Spheres 20 µm to 120 µm in diameter were isolated by wet sieving (Newark Wire Co., Newark, NJ), washed three times with water by centrifugation, then lyophilized to dryness. A separate group of 10% (w/w) bupivacaine microspheres were produced with PLGA₂₀. Twenty milligrams of bupivacaine and 180 mg of PLGA₂₀ were dissolved in 5 ml methylene chloride. The mixture was treated as above except that a) homogenization was performed at 9000 rpm, and b) following rotary evaporation the particles were collected in an Erlenmeyer-flask (rather than in sieves). The purpose of these changes in method was to produce microspheres that were comparable to the LPSPs in size. Particle size was determined with a Coulter Multisizer (Coulter Electronics Ltd., Luton, U.K.).

Animal Care

Young adult male Sprague-Dawley rats weighing 310 - 420 g each were obtained from Taconic Farms (Germantown, NY), and housed in groups in a 6 AM - 6 PM light-dark cycle. Animals were cared for in compliance with protocols

approved by the Animal Care and Use Committee at the Massachusetts Institute of Technology. NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) were observed. Rats were only injected once.

Perineural Injection Technique

- 5 Nerve block injections were performed via a 20 gauge needle under halothane-oxygen anesthesia as described (Kohane *et al.* "Sciatic nerve blockade with lipid-protein-sugar particles containing bupivacaine" *Pharm. Res.* 2000 (in press); incorporated herein by reference). In brief, each rat was injected with a suspension of 75 mg of LPSPs or microspheres suspended in 0.6 ml of 1% sodium
- 10 carboxymethyl cellulose, 0.1% Tween 80 (Castillo *et al.* "Glucocorticoids prolong rat sciatic nerve blockade in vivo from bupivacaine microspheres" *Anesthesiology* 85:1157-66, 1996; Curley *et al.* "Prolonged regional nerve blockade. Injectable biodegradable bupivacaine/polyester microspheres" *Anesthesiology* 84:1401-1410, 1996; Drager *et al.* "Prolonged intercostal nerve blockade in sheep using controlled
- 15 release bupivacaine and dexamethasone from polyester microspheres" *Anesthesiology* 89: 969-979, 1998; each of which is incorporated herein by reference) after gentle agitation (< 5 sec) in preparation for injection. The presence of nerve block (and therefore proper location of the injected particles) was confirmed by hotplate testing (Kohane *et al.* "Sciatic nerve blockade with lipid-protein-sugar particles containing
- 20 bupivacaine" *Pharm. Res.* 2000 (in press); incorporated herein by reference) in all animals, except those injected with blank (no bupivacaine) particles.

Scoring of autotomy and touch-evoked agitation

Each rat was scored for daily autotomy (Wall *et al.* "Autotomy following peripheral nerve lesions: experimental anaesthesia dolorosa" *Pain* 7:103-111, 1979;

incorporated herein by reference) as follows. One point was given for mutilation of one or more nails on the hindpaw. An additional point was assigned for each distal (up to 5 points) and proximal (up to 5 points) half-digit attacked by the rat. Thus the score could range from 0 to 11. Rats who achieved a score of 11 were sacrificed.

- 5 The site of injection and ipsilateral leg of each rat was lightly palpated for touch-evoked agitation at hourly intervals for the first eight hours, then daily, using the following scoring system (Yanez *et al.* "Touch-evoked agitation produced by spinally administered phospholipid emulsion and liposomes in rats" *Anesthesiology* 82:1189-1198, 1995). Normal behavior to touch = 0; occasional squeaking, mild
10 agitation to touch = 1; spontaneous agitation or squeaking or severe agitation to touch = 2.

Rat Sciatic Nerve Dissection

- Under deep halothane/oxygen general anesthesia, the sciatic nerve was exposed by a longitudinal incision on the lateral thigh, followed by careful separation
15 of the muscles of the anterior and posterior thigh. The wound was extended proximally until the entire area of injection (the area posteromedial to the greater trochanter) was fully revealed. The nerve was then cut above the greater trochanter and at the trifurcation and placed into 4% formaldehyde at 4 °C. Intraperitoneal pentobarbital (100 mg/kg) was administered following removal of the nerve.

- 20 The dissector was blinded as to which type of particle each rat had been injected with. At the time of dissection, the degree to which the tissues surrounding the nerve were matted together was scored as follows: "0": tissue planes obvious and easily separated, "1": tissue planes obvious but separated with some difficulty, "2": tissue planes effaced and separated with some difficulty, "3": tissue planes completely

obliterated, could not separate surrounding tissues from nerve without cutting through them.

Histological Preparations

Embedding, sectioning, and staining with hematoxylin/eosin of dissected
5 nerves were performed using standard techniques. The amount of inflammation was estimated by cell counting in the most inflamed areas of the sections (selected under light microscopy). The observer was blinded as to the type of particles present in each sample. Cell counts were performed on digitized images obtained using light microscopy at 400X magnification. Image size was calculated using a calibration
10 micrometer. The area of inflammation in each dissected nerve was estimated by measuring the long and short axes of the mass and assuming a generally rectangular shape.

Statistical Analysis

Neurobehavioral data are reported as means with standard deviations.
15 Comparisons between groups of such data were made using Student's t-test. Non-parametric data (dissection scores, presence vs. absence of polymer residue) and data that were not normally distributed (area of inflammation) were compared using the Mann-Whitney U-test.

20 **Results**

Characteristics of LPSPs and PLGA microspheres

The data relating to the production and characterization of the LPSPs and PLGA₁₁₀ microspheres has been described in Example 1 and elsewhere (Kohane *et al.* "Sciatic nerve blockade with lipid-protein-sugar particles containing bupivacaine"

Pharm. Res. 2000 (in press); incorporated herein by reference). Relevant aspects are summarized in Table 2, together with data on PLGA₂₀ microspheres.

Table 2. Characteristics of particles

Particle Type	Composition		Median particle diameter (μm)
	Polymer	Bupivacaine ¹ %(w/w)	
LPSP ²	- ³	10%	4.4 ± 0.4
PLGA ⁴	PLGA ₁₁₀	50%	59 ± 12
	PLGA ₂₀	10%	3.6 ± 0.2

- 5 ¹ Theoretical loading. Actual loading was approximately 80% of this value (Kohane *et al.* "Sciatic nerve blockade with lipid-protein-sugar particles containing bupivacaine" *Pharm. Res.* 2000 (in press); incorporated herein by reference).

² Lipid proteins sugar particles.

³ The excipients are dipalmitoylphosphatidylcholine, albumin, and lactose.

- 10 ⁴ Poly (lactic-co-glycolic) acid. The subscript refers to the molecular weight. See Materials for further details.

Tissue reaction

Groups of rats were injected at the sciatic nerve with 10% (w/w) bupivacaine

- 15 LPSPs or 50% (w/w) PLGA₁₁₀ microspheres. The sciatic nerves were removed 4 days (n = 4), 2 weeks (n = 6), or 7 months (n = 4, LPSP; n = 5, PLGA₁₁₀) after injection and processed for histology, together with adherent tissues.

General observations. Fig. 5 shows the histological appearance of a control nerve sciatic nerve. Fig. 6 is a representative photomicrograph demonstrating the

- 20 delivery of particles (in this case PLGA₁₁₀ microspheres) to the vicinity of the sciatic nerve. If detectable, both particle types were localized in this manner at all time points (except as described below). In general (and at all time points examined), the inflammatory response to PLGA₁₁₀ microspheres was confined to clearly demarcated

pockets. Inflammation from LPSP was found more diffusely throughout the tissues. In general, neither type of LPSPs nor PLGA₁₁₀ microspheres were found within the perineurium. There was no clinical or histological evidence of infection in any of the animals at any time point.

- 5 Tissue reaction four days after injection. All rats injected with PLGA₁₁₀ microspheres had large, firm, slightly gritty deposits of particles in discrete globules around the nerve. Three out of four rats that received LPSP had visible deposits of particles, which were much smaller than the PLGA₁₁₀ deposits and were soft to the touch. There was no statistically significant difference between the PLGA₁₁₀ and
- 10 LPSP groups in dissection scores, presence of polymer residue, or cell density (Figure 7). The area of inflammation was not quantitated because it was too large and amorphous in the PLGA group to be dissected *en bloc*. Both groups had large areas of inflammation and high cell densities on light microscopy, there were marked histological differences. As demonstrated in Figure 8, the PLGA₁₁₀ group showed a
- 15 foreign-body-type granulomatous reaction with multinucleated giant cells surrounding the microspheres (which appear as empty circles 50 to 90 μ m across, some with traces of polymer remaining). The LPSP group showed mostly acute inflammation with lymphocytes and macrophages and occasional neutrophils and foamy macrophages. LPSP were not discernible as discrete particles, but as an area of diffuse pink staining.
- 20 In some animals in both groups, inflammation also involved adjacent muscle and perineural adipose tissue, with necrotic muscle fibers, myophagocytosis, and myocyte regeneration—all signs of acute muscle injury. Necrosis was associated with acute inflammation, with neutrophils forming small sterile microabscesses.

Tissue reaction two weeks after injection. Deposits of microspheres were still

found in all rats injected with PLGA₁₁₀ microspheres; these were often several millimeters across. In comparison, a very small amount of residue (approximately 0.5 mm by 3 mm) was found in only one of the rats injected with LPSPs. This difference in incidence was statistically significant ($p = 0.005$). PLGA₁₁₀ microspheres were
5 surrounded with a large dense granulomatous foreign body reaction (Figure 9a) with lymphocytes and macrophages, while LPSPs had a small loose, predominantly lymphocytic infiltrate (Figure 9b).

Microscopic examination of histological preparations from rats injected with PLGA₁₁₀ microspheres revealed 4800 ± 1900 cells/mm², which was 3.4 times more (p
10 $= 0.006$) than the 1400 ± 600 cells/mm² seen in those injected with LPSP (Figure 7). The area of inflammation was also much larger in the PLGA₁₁₀ group (Figure 7, $p = 0.01$). One PLGA₁₁₀-treated nerve showed neutrophils and active myositis. In another PLGA₁₁₀-treated rat, a massive cavitory mass was found at the injection site, with a pronounced granulomatous reaction and a large degree of axonal degeneration,
15 on the side of the nerve facing the granuloma.

Tissue reaction eight weeks after injection. An additional group who received PLGA₁₁₀ microspheres was harvested eight weeks after injection ($n = 5$). All five rats showed large amounts of polymer residue. All rats had a dissection score of 2. The histological appearance was comparable to that seen at 2 weeks, with a cell density of
20 3500 ± 1800 cells/mm². There was no counterpart group for LPSPs because there was already almost no particle mass to follow at two weeks. (Note the very small size of the inflammatory masses in the LPSP group at 2 weeks, Figure 7c). The cell count and incidence of residue in the PLGA₁₁₀ group was higher at 8 weeks than the corresponding values had been in the LPSP group at 2 weeks (six weeks earlier).

Tissue reaction seven months (210 days) after injection. All of the dissections of LPSP-injected rats ($n = 4$) were scored as "0". In the PLGA₁₁₀ group ($n = 5$), two had a score of "1", and one had a score of "2" ($p = 0.08$). No pockets of polymer residue were visible at dissection in either group. Microscopic examination of most samples from both groups at this time point were felt to be either entirely normal, or to have slightly increased cellularity with a loose architecture, suggesting the possibility of edema, with several lymphocytes. One sample from the PLGA₁₁₀ groups showed a small foreign body reaction around a piece of extraneous material. There was no statistically significant difference in cell density between groups, nor did either represent a significant increase over the cellularity of control nerves (370 ± 40 cells/mm², $p = \text{n.s.}$ vs. both particle groups). There was no obvious area of inflammation to measure in either group.

In order to assess the contribution of particle size to the differences noted between LPSPs and PLGA microspheres, four rats were injected with PLGA₂₀ microspheres 3.6 ± 0.2 μm in diameter (vs. 4.4 ± 0.4 μm for the LPSPs) loaded 10% (w/w) with bupivacaine. (In order to further minimize the dwell time of the microspheres we used PLGA₂₀, a polymer that has a much lower molecular weight and higher proportion of glycolic to lactic acid monomers than PLGA₁₁₀). Two weeks after injection, the median dissection score was 3, all four rats had large pockets of polymer residue, and the cell density was 7356 ± 1604 cells/mm² ($p = 0.06$ compared to PLGA₁₁₀ group). The outlines of microspheres were still visible on histology. There were many macrophages and lymphocytes, and occasional giant cells (a much lower incidence of the latter than with the PLGA₁₁₀ microspheres).

Other findings on dissection

In some animals injected with PLGA₁₁₀ microspheres, particles were found at locations where they were not intentionally placed. In the group dissected four days after injection, one of the animals had a continuous cord of particles that extended
5 almost to the knee from the site of injection, and two others had visible particle residue tracking up into the subcutaneous tissue. Similar findings occurred in the group dissected two weeks after injection. In one rat, microspheres were found within the nerve, beneath the perineural sheath (Figure 10a), with infiltration of inflammatory cells. In another rat, a mass of microspheres surrounded by
10 inflammation, measuring 8 mm x 0.4 mm x 2 mm was found 2.5 cm distal to the site of injection along the course of the sciatic nerve, near the knee (Figure 10b). There were no similar findings in rats injected with LPSPs.

Touch-evoked agitation, distress, and autotomy scoring

All rats (PLGA₁₁₀-, PLGA₂₀-, and LPSP-treated groups) were palpated at the
15 site of injection and on the ipsilateral leg as per Methods. None of the rats in any of the groups appeared distressed by this maneuver (score = 0). Furthermore, no rat had an autotomy score above zero. All the rats appeared well groomed, and continued to gain weight throughout the duration of the experiment.

All of the rats in this study had full recovery of sensory and motor function in
20 the injected extremity when the local anesthetic effect of the microparticles resolved (data not shown), and none had any detectable signs of long-term functional deficits or pain-related behavior.

Discussion

The primary focus of this study was to compare the tissue reactions to LPSPs and PLGA microspheres that had previously been shown to cause approximately equivalent durations of sciatic sensory nerve blockade in the rat (Kohane *et al.*

- 5 “Sciatic nerve blockade with lipid-protein-sugar particles containing bupivacaine” *Pharm. Res.* 2000 (in press); incorporated herein by reference).

There were striking differences in the degree of inflammatory response to the LPSP and PLGA particles at two weeks after injection. There was only a mild patchy lymphocytic infiltrate around nerves where LPSPs had been injected. In contrast, the

- 10 PLGA₁₁₀ microspheres produced a tissue response that was consistent with the observations of other investigators (Drager *et al.* “Prolonged intercostal nerve blockade in sheep using controlled release bupivacaine and dexamethasone from polyester microspheres” *Anesthesiology* 89: 969-979, 1998; van der Elst *et al.* “Bone tissue response to biodegradable polymers used for intramedullary fracture fixation: A
- 15 long-term in vivo study in sheep femora” *Biomaterials* 20: 121-128, 1999; each of which is incorporated herein by reference), with pronounced inflammation at least as far out as 8 weeks (n = 5, data not shown). Traces of the reaction to PLGA₁₁₀ were seen as far out as 7 months after injection. Since particle residue was noted for a much longer time in rats injected with PLGA microspheres than LPSPs, and the
- 20 durations of anesthetic effect were similar, the ratio of duration of therapeutic effect to duration of polymer residue is better for the LPSPs. This suggests that the latter may be more suitable for repeated injections, particularly at the same site.

Although the long-term biocompatibility of the LPSPs was superior to that of the PLGA microspheres, this may not be related to the fact that the excipients were

naturally occurring in the human body. This is shown by the fact that there was marked inflammation at 4 days post-injection. Instead, the improved long-term biocompatibility may be due to the much shorter dwell time of the particles in the tissue.

- 5 The shorter duration of inflammation from LPSPs could be due to their being much smaller than the PLGA₁₁₀ microspheres and therefore more easily taken up and removed by leukocytes (Tabata *et al.* "Phagocytosis of polymer microspheres by macrophages" *Adv. Polymer Sci.* 94:107-141; 1990; incorporated herein by reference). The fact that 3.6 μ m diameter PLGA₂₀ microspheres also showed
- 10 prolonged inflammation argues against a size difference being the sole explanation. The differences in duration of inflammatory response may therefore be partly due to the materials used *per se*. (Note that we used a relatively rapidly degrading PLGA in the smaller microspheres; nevertheless particles were still visible on histology).

- The nature of the tissue response elicited by the LPSPs (acute inflammation) at
- 15 all time points was different from that seen with PLGA₁₁₀ microspheres (granulomatous foreign body reaction). It is possible that this difference is due to the difference in particle size, as suggested by the fact that the 3.6 μ m PLGA₂₀ microspheres showed less of a giant cell foreign body type reaction than was seen with the larger microspheres. This is consistent with fact that macrophages tend to
- 20 ingest small particles, but tend to form multinucleated giant cells to surround objects larger than themselves.

It bears mentioning that the tissue reaction to both particle types was not due to the encapsulated bupivacaine. Blank (no drug) LPSPs and PLGA₁₁₀ microspheres (n = 4 each) produced the same qualitative and quantitative tissue effects seen with

drug-loaded particles two weeks after injection.

Any material that is injected blindly (*i.e.*, not under direct visualization) into the body has the potential for being injected at a site other than the intended target, or of being injected at the correct location but being pushed away by the force of
5 injection, or of migrating even if deposited at the correct location. These possibilities were demonstrated in the rat in which inadvertent intraneural injection occurred, and in the ones in which large inflammatory masses were found tracking to the knee. Those cases point out the potential hazards when a) particles are injected either near a vital or sensitive structure or b) the site of injection itself does not confine the
10 particles to a locale effectively. Although it is probably equally likely for such events to occur with either type of particle, particles which disappear more rapidly from the tissues and that cause less long-term inflammation may be safer. Given the potentially severe sequelae of having an inflammatory mass extending into a nerve, it will probably be advisable to take special precautions (nerve stimulator, radiological
15 guidance) when performing blocks with such particles at sites where a major nerve could be hit.

None of the rats injected with either type of particle showed any neurological deficits, even where there was marked inflammation or intraneural microspheres. The mean thermal latency prior to sacrifice was comparable to that in pristine rats. None
20 of the rats showed any "touch-evoked agitation" or autotomy.

Example 3 – Formulation with Anticonvulsants, Vasodilators, Proteins, Lipids, and
Glycosaminoglycans

Lipid-protein-sugar particles containing various agents were prepared as
5 described above in Example 1. These formulations included anticonvulsants,
vasodilators, proteins, lipids, and glycosaminoglycans. The particles were prepared
with varying amounts of the agent to be delivered. Drug loading typically ranged
from 10% (w/w) to 80% (w/w). After preparation of the loaded particles, the release
of the agent from the LPSPs was then studied under physiological conditions using *in*
10 *vitro* assays known in the art.

With respect of anticonvulsants, muscimol was loaded into the LPSPs at 1 to
20 micrograms of drug per milligram of particle. LPSPs loaded with muscimol were
prepared, and the release of muscimol was studied in comparison to free muscimol in
an *in vitro* dialysis assay as described above in the section, entitled “*In vitro* release of
15 bupivacaine from microparticles,” of Example 1. LPSPs were also prepared
containing 20% (w/w) of diphenylhydantoin.

LPSPs with vasodilators were also prepared. For example, LPSPs loaded with
10% (w/w) to 80% (w/w) nifedipine were prepared using the method described above.
Interestingly, conventional PLGA poly(lactic-co-glycolic) acid microspheres loaded
20 with over 20% nifedipine cannot be prepared. The LPSPs loaded with nifedipine
were then used to study the release kinetics of the nifedipine in an *in vitro* model by
placing the loaded LPSPs in an Eppendorf tube with phosphate buffered saline (PBS)
solution and determining nifedipine release.

Proteins were also used as the agent to be delivered by the LPSPs. LPSPs

loaded with FITC-labeled albumin (10-50% (w/w)), rhodamine-labeled lactalbumin (10-50% (w/w)), and glucose oxidase (10% (w/w)) were prepared. Since it is important to be able to deliver an enzyme which retains its catalytic activity, it was shown with the LPSPs containing glucose oxidase that the encapsulated glucose oxidase could still metabolize its substrate using a colorimetric assay.

Lipids were also encapsulated in LPSPs. Phospholipids derivatized with polyethylene glycol (PEG) were loaded into particles ranging from 10% (w/w) to 60% (w/w). Also, glycosaminoglycans, such as chondroitin sulfate and hyaluronic acid, were encapsulated in LPSPs.

10

Other Embodiments

The foregoing has been a description of certain non-limiting preferred embodiments of the invention. Those of ordinary skill in the art will appreciate that various changes and modifications to this description may be made without departing from the spirit or scope of the present invention, as defined in the following claims.

15

Claims

What is claimed is:

- 5 1. A pharmaceutical composition comprising microparticles of an agent encapsulated in a matrix comprising lipid, protein, and sugar.
2. A pharmaceutical composition comprising microparticles of an agent encapsulated in a matrix, wherein the matrix comprises at least three components
10 selected from the group consisting of lipid, protein, sugar, and synthetic polymer.
3. A pharmaceutical composition comprising microparticles of an agent encapsulated in a matrix, wherein the matrix comprises at least two components selected from the group consisting of lipid, protein, sugar, and synthetic polymer.
15
4. A pharmaceutical composition comprising microparticles of an agent encapsulated in a matrix comprising lipid and protein.
5. A pharmaceutical composition comprising microparticles of an agent
20 encapsulated in a matrix comprising lipid and sugar.
6. A pharmaceutical composition comprising microparticles of an agent encapsulated in a matrix comprising protein and sugar.

7. The pharmaceutical composition of claim 1 wherein the agent is a therapeutic agent.
8. The pharmaceutical composition of claim 1 wherein the agent is a local
5 anesthetic.
9. The pharmaceutical composition of claim 1 wherein the agent is selected from the group consisting of procaine, lidocaine, dibucaine, tetracaine, bupivacaine, mepivacaine, and articaine.
- 10
10. The pharmaceutical composition of claim 1 wherein the agent is bupivacaine.
11. The pharmaceutical composition of claim 1 wherein the agent is an anticonvulsant.
- 15
12. The pharmaceutical composition of claim 1 wherein the agent is a vasodilator.
13. The pharmaceutical composition of claim 1 wherein the agent is a protein.
- 20
14. The pharmaceutical composition of claim 1 wherein the agent is a lipid.
15. The pharmaceutical composition of claim 1 wherein the agent is a glycosaminoglycan.

16. The pharmaceutical composition of claim 1 wherein the agent is a diagnostic agent.
17. The pharmaceutical composition of claim 1 wherein the agent is a
5 prophylactic agent.
18. The pharmaceutical composition of claim 1 wherein the lipid is a naturally occurring
lipid.
- 10 19. The pharmaceutical composition of claim 1 wherein the lipid is an emulsifier.
20. The pharmaceutical composition of claim 1 wherein the lipid is a surfactant.
- 15 21. The pharmaceutical composition of claim 1 wherein the lipid is positively charged.
22. The pharmaceutical composition of claim 1 wherein the lipid is negatively charged.
- 20 23. The pharmaceutical composition of claim 1 wherein the lipid has no charge.
24. The pharmaceutical composition of claim 1 wherein the lipid is a phosphatidylcholine.

25. The pharmaceutical composition of claim 1 wherein the lipid is dipalmitoylphosphatidylcholine (DPPC).
- 5 26. The pharmaceutical composition of claim 1 wherein the lipid is polyvinyl alcohol.
27. The pharmaceutical composition of claim 1 wherein the lipid is a phospholipid.
- 10 28. The pharmaceutical composition of claim 1 wherein the lipid is selected from the groups consisting of phosphoglycerides; phosphatidylcholines; dipalmitoyl phosphatidylcholine (DPPC); dioleoylphosphatidyl ethanolamine (DOPE); dioleyloxypropyltriethylammonium (DOTMA); dioleoylphosphatidylcholine; 15 cholesterol; cholesterol ester; diacylglycerol; diacylglycerolsuccinate; diphosphatidyl glycerol (DPPG); hexanadecanol; fatty alcohols such as polyethylene glycol (PEG); polyoxyethylene-9-lauryl ether; a surface active fatty acid, such as palmitic acid or oleic acid; fatty acids; fatty acid amides; sorbitan trioleate (Span 85) glycocholate; surfactin; a poloxomer; a sorbitan fatty acid ester such as sorbitan trioleate; lecithin; 20 lysolecithin; phosphatidylserine; phosphatidylinositol; sphingomyelin; phosphatidylethanolamine (cephalin); cardiolipin; phosphatidic acid; cerebroside; dicetylphosphate; dipalmitoylphosphatidylglycerol; stearylamine; dodecylamine; hexadecyl-amine; acetyl palmitate; glycerol ricinoleate; hexadecyl stearate; isopropyl

myristate; tyloxapol; poly(ethylene glycol)5000-phosphatidylethanolamine; and phospholipids.

29. The pharmaceutical composition of claim 1, wherein the lipid is a derivatized
5 lipid.

30. The pharmaceutical composition of claim 1 wherein the protein is an albumin.

31. The pharmaceutical composition of claim 1 wherein the protein is a whole cell
10 extract.

32. The pharmaceutical composition of claim 1 wherein the protein is an antibody.

33. The pharmaceutical composition of claim 1 wherein the protein is an enzyme.
15

34. The pharmaceutical composition of claim 1 wherein the protein is glucose oxidase.

35. The pharmaceutical composition of claim 1 wherein the protein is insulin.
20

36. The pharmaceutical composition of claim 1 wherein the sugar comprises a mixture of complex and simple sugars.

37. The pharmaceutical composition of claim 1 wherein the sugar is lactose.

38. The pharmaceutical composition of claim 1 wherein the sugar is cellulose.
39. The pharmaceutical composition of claim 1 wherein the sugar is a chemically
5 modified sugar.
40. The pharmaceutical composition of claim 1 wherein the sugar is a
glycosaminoglycan.
- 10 41. The pharmaceutical composition of claim 1 wherein the sugar is dextran.
42. The pharmaceutical composition of claim 1 wherein the sugar is a chemically
modified dextran.
- 15 43. The pharmaceutical composition of claim 1 wherein the sugar is chondroitin
sulfate.
44. The pharmaceutical composition of claim 1 wherein the sugar is a derivatized
sugar.
- 20 45. The pharmaceutical composition of claim 1 wherein the sugar is a chemically
modified sugar.

46. The pharmaceutical composition of claim 1 wherein the sugar is selected from the group consisting of galactose, lactose, glucose, maltose, starches, cellulose and its derivatives, methyl cellulose, carboxymethyl cellulose, fructose, dextran and its derivatives, raffinose, mannitol, xylose, dextrans, glycosaminoglycans, sialic acid, chitosan, hyaluronic acid, and chondroitin sulfate.
47. The pharmaceutical composition of claim 1 wherein the ratio of lipid to protein to sugar is approximately 3:1:1.
48. The pharmaceutical composition of claim 1 wherein the lipid comprises 0-99% of the matrix by weight.
49. The pharmaceutical composition of claim 1 wherein the lipid comprises 3-99% of the matrix by weight.
50. The pharmaceutical composition of claim 1 wherein the lipid comprises 20-60% of the matrix by weight.
51. The pharmaceutical composition of claim 1 wherein the protein comprises 0-95% of the matrix by weight.
52. The pharmaceutical composition of claim 1 wherein the protein comprises 10-30% of the matrix by weight.

53. The pharmaceutical composition of claim 1 wherein the protein comprises 1-20% of the matrix by weight.
- 5 54. The pharmaceutical composition of claim 1 wherein the sugar comprises 0-60% of the matrix by weight.
55. The pharmaceutical composition of claim 1 wherein the sugar comprises 0.5%-50% of the matrix by weight.
- 10 56. The pharmaceutical composition of claim 1 wherein the sugar comprises 10-30% of the matrix by weight.
57. The pharmaceutical composition of claim 1 wherein the microparticles are less
15 than 50 micrometers in diameter.
58. The pharmaceutical composition of claim 1 wherein the microparticles are less than 10 micrometers in diameter.
- 20 59. The pharmaceutical composition of claim 1 wherein the microparticles are less than 5 micrometers in diameter.
60. The pharmaceutical composition of claim 1 wherein the microparticles are less than 1 micrometer in diameter.

61. The pharmaceutical composition of claim 1 wherein the microparticles are less than 500 nanometers in diameter.
- 5 62. A method of preparing microparticles comprising an agent encapsulated in a lipid-protein-sugar matrix, the method comprising steps of:
- providing an agent;
- contacting the agent with a lipid, a protein, and a sugar; and
- spray drying mixture of the agent, the lipid, the protein, and the sugar to make
- 10 microparticles.
63. A method of administering an agent, the method comprising steps of:
- providing a patient;
- providing microparticles of an agent encapsulated in a lipid-protein-sugar
- 15 matrix; and
- administering the microparticles to the patient.
64. The method of claim 63 wherein the step of administering comprises injecting the microparticles into the patient.
- 20 65. The method of claim 63 wherein the step of administering comprises placing the microparticles in a body cavity of the patient.
66. A method of administering a nerve block, the method comprising steps of:

providing a patient;

providing microparticles of a local anesthetic encapsulated in a lipid-protein-sugar matrix; and

injecting the microparticles into the patient near a nerve to be anesthetized.

5

67. The method of claim 66 wherein the nerve is a sciatic nerve.

68. The method of claim 66 wherein the nerve is a femoral nerve.

10 69. The method of claim 66 wherein the nerve is a inferior alveolar nerve.

70. The method of claim 66 wherein the nerve is a nerve of the brachial plexus.

71. The method of claim 66 wherein the nerve is an intercostal nerve.

15

72. The method of claim 66 wherein the local anesthetic is bupivacaine.

73. A method of immunizing an individual, the method comprising steps of:
providing an individual;

20 providing microparticles comprising a prophylactic agent encapsulated in a
lipid-protein-sugar matrix; and

delivering an effective amount of the microparticles to the individual to
stimulate an immune response.

74. The method of claim 73 wherein the prophylactic agent is an antigen.
75. The method of claim 73 wherein the prophylactic agent is a protein.
- 5 76. The method of claim 73 wherein the prophylactic agent is selected from the group consisting of bacterial antigens, viral antigens, protozoan antigens, and parasite antigens.
77. The method of claim 73 wherein the microparticles further comprise an
10 adjuvant.
78. The method of claim 73 wherein the microparticles are at least 5 micrometers in diameter.
- 15 79. The method of claim 73 wherein the microparticles are less than 5 micrometers in diameter.

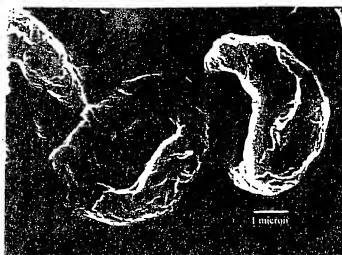


Fig. 1

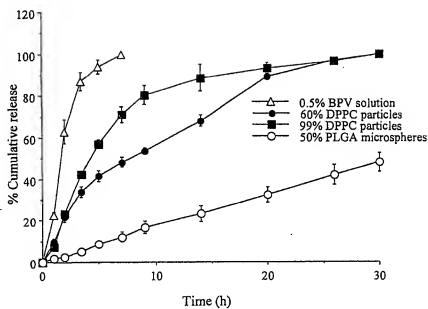


Fig 2

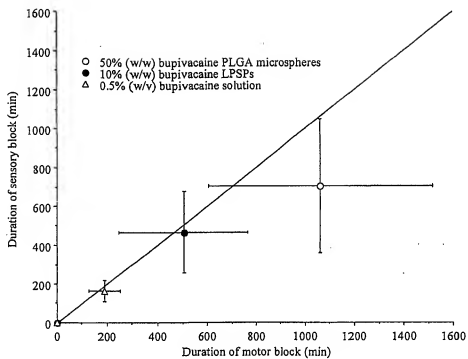


Fig 3

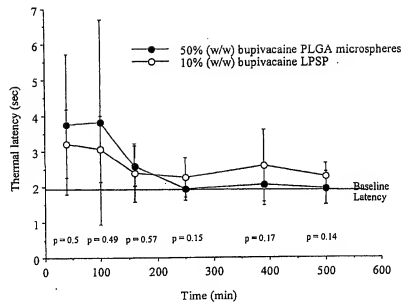


Fig 4

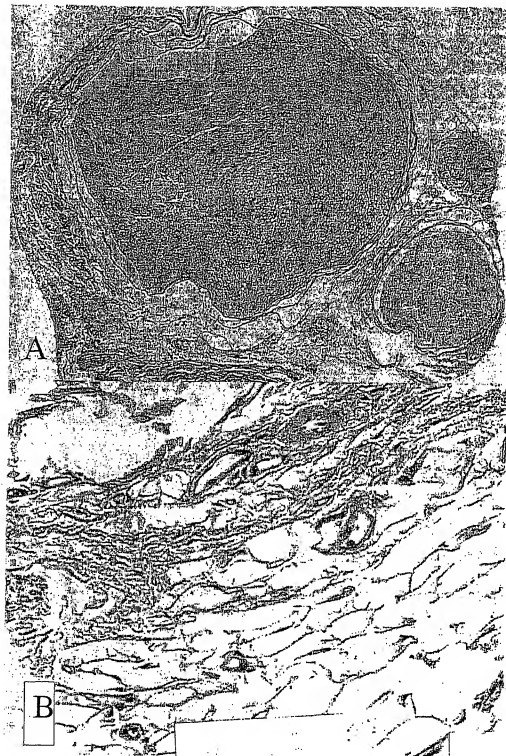


Fig 5

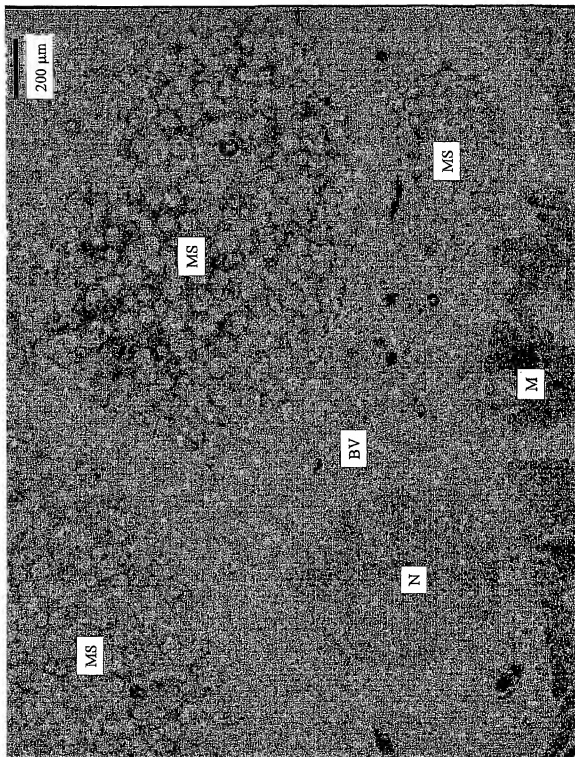


Fig. 6

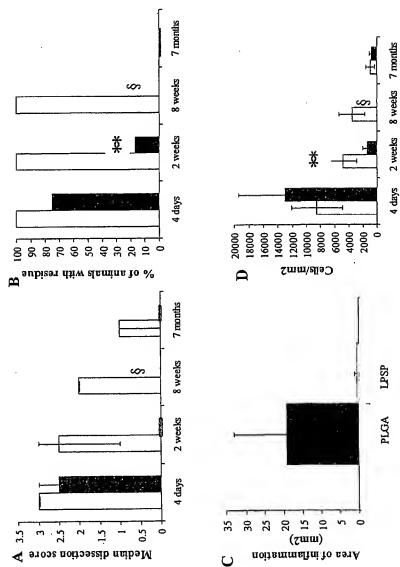
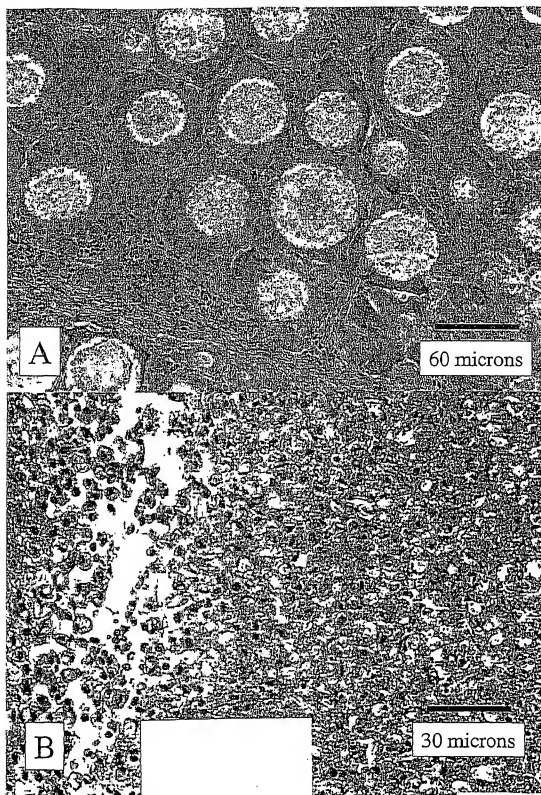
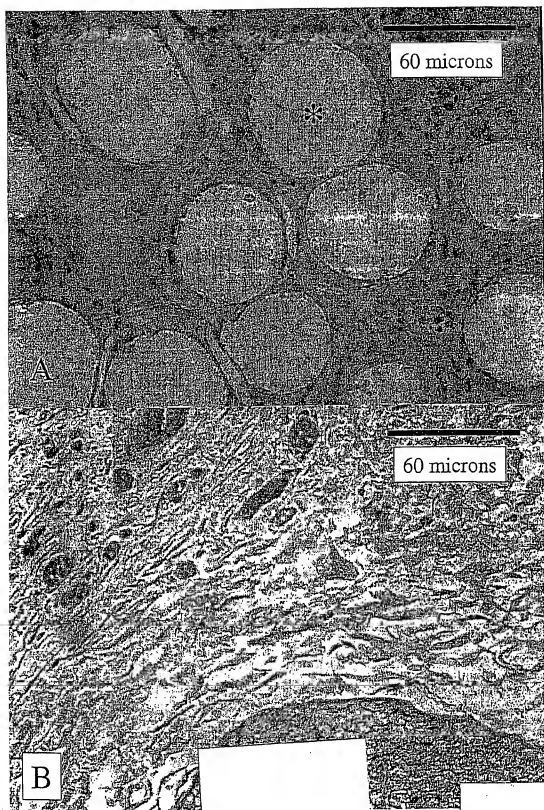


Fig. 7

*Fig. 8*



FD.9

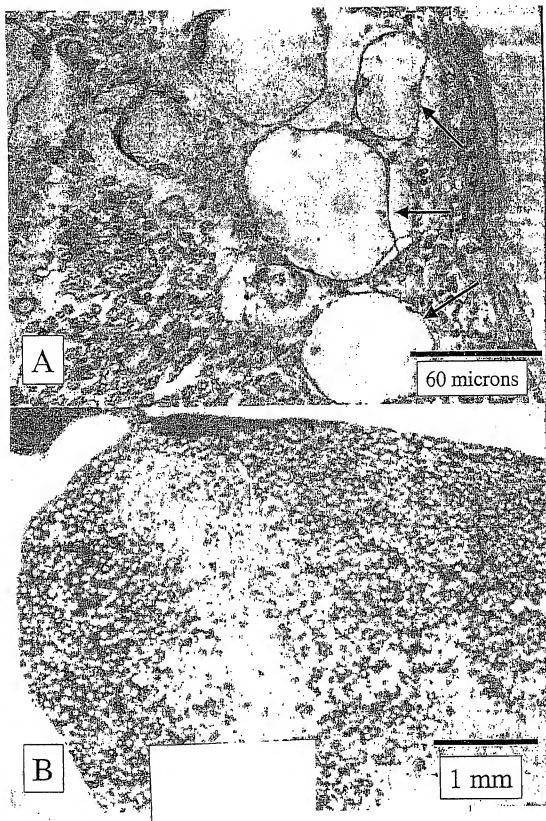


Fig. 10

Fig.11 - Muscimol

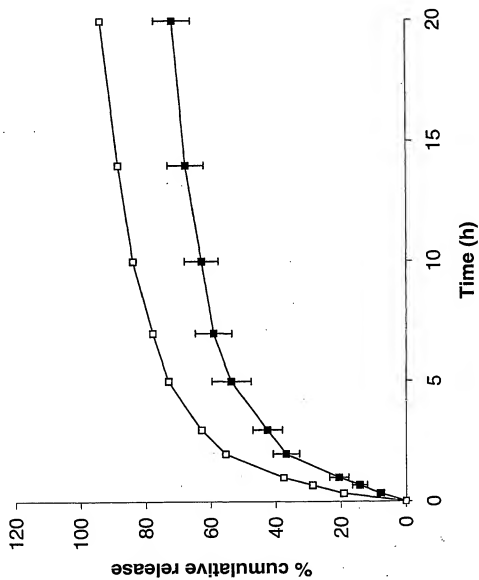


Fig.12 - Nifedipine Particle Release